University of Alberta

The Effects of Isoproterenol on Energy Substrate Metabolism and Function in Newborn Hearts

By

Chad Romney Lund

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Pharmacology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, **The Effects of Isoproterenol on Energy Substrate Metabolism and Function in Newborn Hearts,** submitted by **Chad Romney Lund** in partial fulfillment of the requirements for the degree of Master of Science.

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Dedication

The work and effort embodied in this thesis, and lessons learned over these two years of my life, are dedicated to those who taught me everything . . .
my father, my mother and my brother.

Abstract

This thesis examined the effects of inotropic treatment on energy substrate metabolism and function of newborn hearts. Energy substrate metabolism of newborn rabbit hearts were examined under aerobic conditions with low concentrations of fatty acids (0.4 mM palmitate) in the presence or absence of isoproterenol. It was observed that fatty acid oxidation remains the major source of energy for the myocardium after inotropic administration. I hypothesized that these high rates of fatty acid oxidation would result in poor recovery from an ischemic insult. To test this hypothesis, energy substrate metabolism of newborn rabbit hearts were examined through ischemia and reperfusion with high concentrations of fatty acids (1.2 mM palmitate), in the presence of absence of isoproterenol. It was concluded that, while fatty acid oxidation remains the major source of energy in newborn hearts in the presence of isoproterenol before and after an ischemic insult, it does not result in worse recovery of function than control hearts.

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Abbreviations

ACC	acetyl CoA carboxylase
ADP	adenosine diphosphate
AMARA	synthetic peptide (AMARASAAALARRR) substrate for AMPK
AMP	adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
AR	adrenoreceptor
ATP	adenosine triphosphate
BSA	bovine serum albumin
CO_2	carbon dioxide
CoA	co-enzyme A
CPT 1	carnitine palmitoyl transferase 1
DCA	dichloroacetate
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethylene glycol-bis(b-aminoethyl ether)N,N,N',N'-tetraacetic acid
FADH	flavin adenine dinucleotide (reduced)
GADPH	glyceraldehyde 3-phosphate dehydrogenase
HPLC	high performance liquid chromatography
MOPS	3-[N-morpholino]propane sulfonic acid
NADH	nicotinamide adenine dinucleotide (reduced)
O_2	oxygen
PCA	perchloric acid
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PFK	phosphofructokinase
PMSF	phenylmethylsulfonyl floride
PKA	protein kinase A
TCA	tricarboxylic acid
Tris	Tris(hydroxymethyl)aminomethane
SR	sarcoplasmic reticulum

Chapter I

Introduction

Energy substrate metabolism in the heart is intimately related to heart function. Under ideal conditions, the myocardium will be provided with those substrates that will allow it to function effectively. However, in certain conditions, serum levels of energy substrates may be such that heart metabolism is altered and function will ultimately suffer. Cardiac surgery provides such an environment in which cardiac function may be jeopardized. Prior to surgery heparin administration [reviewed in Tan (1)] and endogenous catecholamine (2) release will increase plasma levels of fatty acids. The severe ischemia experienced during surgery generally permits only anaerobic metabolism, and therefore tissue acidosis occurs. Upon reperfusion, the myocardium is subjected to greater than normal levels of fatty acids and the resulting high rates of fatty acid oxidation can exacerbate ischemic injury [reviewed in Lopaschuk (3)].

Surgical correction of congenital heart defects (CHD) necessitates that the myocardium must endure a period of ischemia. While it is generally accepted that inotropic support in the peri-ischemic period is best avoided, many infants will require inotropic support after, and perhaps before, surgical intervention. The administration of exogenous inotropes, in light of their potent effects on cardiac function and cardiac metabolism, in addition to increasing levels of fatty acids, is

cause for concern. The intimate relationship between heart metabolism of energy substrates and cardiac function may become unbalanced.

Although research has been performed in adult heart models, no examination of the effects of inotropes on energy substrate metabolism in the newborn heart has occurred. It is essential to assess the newborn heart independently, as the neonatal myocardium is in a process of maturation. At this point, whether or not pre- or post-operative operative inotropic support may force the neonatal myocardium to adopt a profile of energy substrate metabolism that is unfavorable, in the context of ischemia, is not known.

1) *Myocardial Energy Substrate Metabolism*

1.1) *Overview*

Under basal conditions, the mature myocardium is an omnivore, metabolizing lipids, carbohydrates, ketone bodies and amino acids. While the heart is capable of metabolizing amino acids and ketone bodies, the majority of energy that the mature heart produces is provided by lipids (60% - 70%) and carbohydrates (glucose, lactate). In the myocardium, lipids, as fatty acids, are metabolized via β oxidation, glucose via glycolysis and pyruvate oxidation, and lactate via pyruvate oxidation (Figure I.1).

1.2) *Glycolysis*

Primary metabolism of glucose is an anaerobic process common to all living organisms (Figure I.2). In the process of glycolysis, one mole of glucose is metabolized to 2 moles of pyruvate producing 2 moles of ATP. The metabolism

of glucose to ATP and pyruvate is orchestrated by a series of cytosolic enzymes rate-limited by phosphofructokinase (PFK). Pyruvate is converted to lactate via lactate dehydrogenase, a reaction in which lactate is the favored product, and 2 H^+ are produced via ATP hydrolysis.

1.3) *Glucose Oxidation*

Glucose oxidation only occurs in the presence of oxygen and is dependent on glycolysis and lactate for supply of pyruvate. Lactate extracted from the blood is converted to pyruvate via lactate dehydrogenase in the cytosol. Pyruvate is taken up into the mitochondria and converted to acetyl CoA by pyruvate dehydrogenase (PDH). Acetyl CoA enters the tricarboxylic acid (TCA) cycle (Figure I.3) and reacts with oxaloacetate to form citrate. Following a series of reactions, oxaloacetate is regenerated to react with another molecule of acetyl CoA. Energy, ATP and reducing agents, produced by the TCA cycle are 4 ATP, 2 $FADH_2$ and 10 NADH per molecule of glucose. The reducing agents, $FADH_2$ and NADH, then donate their electrons to the electron acceptors of the electron transport chain.

1.4) *β Oxidation*

Fatty acids are metabolized to acetyl CoA via sequential oxidation of the β carbon (Figure I.4). As fatty acids vary in the length of their carbon tails, the quantity of acetyl CoA, $FADH_2$ and NADH produced will vary accordingly. Generally, for every cycle of β oxidation one molecule of acetyl CoA, $FADH_2$ and

NADH are generated. However, on the last cycle 2 molecules of acetyl CoA are produced. Thus, for palmitate (C 16) 8 molecules of acetyl CoA and 7 molecules each, of $FADH_2$ and NADH, are generated. Acetyl CoA produced enters the TCA cycle, and reducing agents, $FADH_2$ and NADH, donate their electrons to the electron acceptors of the electron transport chain.

1.5) *Other Substrates*

Amino acids and ketone bodies may also be metabolized to yield ATP, however they are not used readily.

1.6) *Electron Transport Chain*

The $FADH_2$ and NADH contributed from the oxidation of glucose, lactate and fatty acids donate their electrons to electron acceptors, with the terminal electron acceptor being O_2 (Figure I.5). ATP is produced via the return of H^+ through ATP synthase in the intramitochondrial membrane following the pumping of H^+, against the concentration gradient, into the intermembrane space of the mitochondria.

2) *Regulation of Energy Substrate Metabolism*

2.1) *Carbohydrates*

Regulation of carbohydrate metabolism in the myocardium is multi-factorial [reviewed in Stanley *et al.*(4)]. Nutritional status, coronary circulation, inotropic state, and plasma levels of energy substrates and hormones all exert regulatory influences. Movement of glucose from the blood into the cell (glucose uptake) is dependent on the concentration and activity of glucose transporters and

the glucose gradient (interstitial to intracellular glucose). Metabolism of glucose to pyruvate (glycolysis) is primarily controlled by the activity of phosphofructokinase (activated by Ca^{2+}, cAMP) and the availability of NAD^{+}. Oxidation of lactate requires transport of lactate across the sarcolemmal membrane by a stereoselective transport protein [monocarboxylate transporter-1 (MCT1)]. Estimates suggest that 80-100% of lactate, which the heart has taken up, is rapidly oxidized. Oxidation of pyruvate is under the primary control of pyruvate dehydrogenase (PDH). Located on the inner side of the mitochondrial membrane, this enzyme complex [pyruvate dehydrogenase complex (PDC)] is under considerable regulation. PDH is activated (dephosphorylated) by pyruvate dehydrogenase phosphatase and inactivated by pyruvate dehydrogenase kinase. The phosphorylation state of and substrate supply for PDH control the conversion of pyruvate to acetyl CoA. In turn, PDH phosphatase (stimulators: Ca^{2+}, Mg^{2+}) and PDH kinase (inhibitors: pyruvate, ADP, DCA; stimulators: acetyl CoA, NADH) are also under regulation.

Glucose that is taken up by cells but is not catabolized can be stored in tissues, including the heart, as glycogen. The enzyme, glycogen synthase, catalyzes the binding of glucose molecules to one another producing the highly branched polymer. The breakdown of glycogen, in response to cellular energy demand and hormonal stimulation, is performed by glycogen phosphorylase. Ischemia and increases in cAMP, via β-AR stimulation, can activate glycogen phosphorylase depleting myocardial glycogen stores.

Insulin is involved in regulation of energy substrate metabolism. Through activating glucose transport into myocytes and, indirectly, by lowering serum levels of fatty acids via inhibition of lipolysis in adipose tissue, insulin directly stimulates carbohydrate metabolism.

2.2) *Fatty Acids*

The high energy demands of the heart are largely met by the oxidation of fatty acids (primarily oleic and palmitic acid) [review in Lopaschuk *et al.* (5)]. The movement of fatty acids into the cell and the mitochondria is a complex process. While the latter process has been delineated fairly extensively, the former is largely unclear [reviewed in Van der Vusse *et al.* (6)]. Once inside the cell, fatty acids destined for β oxidation must enter the mitochondria. The translocation of long-chain fatty acids as fatty acyl CoA is regulated by the enzyme carnitine palmitoyl transferase 1 (CPT 1). Thus, as translocation into the mitochondria is essential for long-chain fatty acid metabolism, CPT 1 is a major regulator of fatty acid oxidation. CPT 1 is, in turn, regulated by malonyl CoA. Malonyl CoA, produced from acetyl CoA via acetyl CoA carboxylase (ACC) in the cytosol, is a potent inhibitor of CPT 1 [reviewed in Lopaschuk *et al.* (7)]. Regulation of ACC is primarily dependent on the availability of acetyl CoA and phosphorylation control by 5'-AMP-activated protein kinase (AMPK) and PKA.

Once inside the mitochondria, fatty acids are metabolized via β oxidation. At sufficient concentrations of fatty acids, β oxidation is primarily regulated by

the energy demand of the cell (acetyl CoA/CoA). It has also been suggested that β oxidation may be regulated by Ca^{2+} levels in the mitochondria via an increase in affinity of several β oxidation enzymes for their respective substrate.

2.3) *Carbohydrate / Fatty Acid Metabolism Inter-regulation*

The presence of high levels of fatty acids in the blood is the primary regulator of carbohydrate oxidation (Figure I.6). Fatty acid concentrations above 0.8 mM will inhibit uptake and oxidation of glucose and lactate while lower fatty acids concentrations (< 0.3 mM) will result in enhanced carbohydrate oxidation. Uptake and metabolism of glucose and lactate are inhibited by high levels of fatty acids primarily via the presence of fatty acid oxidation product (acetyl CoA, NADH, $FADH_2$) [reviewed in Stanley *et al.* (8)]. Glucose oxidation is inhibited by inactivation of PDC by NADH and acetyl CoA. In turn, when plasma levels of fatty acids are low the decrease in high energy products of fatty acid oxidation will remove inhibition of glucose oxidation.

2.4) *Inotropes*

A common effect of all inotropes in the heart is to increase intracellular Ca^{2+}. While cardiac glycosides will evoke a Ca^{2+} increase via interference with sarcolemmal ion exchangers, phosphodiesterase inhibitors and β adrenergic agents evoke the majority of their effects via increasing levels of cAMP. This cAMP phosphorylates and activates PKA. PKA stimulation will result in an increase in the chrono- and inotropic properties of the myocyte via several mechanisms. PKA

phosphorylates Ca^{2+} channels, sarcoplasmic reticulum (SR) phopholamban which activates the SR Ca^{2+}-ATPase and troponin 1. Furthermore, β adrenergic agonists increase heart rate via enhanced phase 4 depolarization of sinoatrial node cells. The net results are a more powerful, rapid contraction. The increase in intracellular Ca^{2+}, following inotropic administration, will stimulate key regulatory enzymes of energy substrate metabolism such as PFK and PDC. In addition, PKA can phosphorylate acetyl CoA carboxylase (ACC) (9), an enzyme involved in the regulation of fatty acid oxidation, while cAMP can stimulate glycolysis directly via activation of PFK (10). Furthermore, in response to increased energy utilization following inotropic treatment, decreases in ATP can stimulate 5'-adenosine monophosphate activated protein kinase (AMPK), another enzyme involved in the regulation of fatty acid oxidation, and decreases in acetyl CoA, NADH can activate PDC.

3) *Energy Substrate Metabolism in the Newborn Heart*

The fetus is largely supplied with carbohydrates, to a lesser degree amino acids, and to a lesser extent fatty acids (Figure I.7) [reviewed in Girard *et al.* (11). Fatty acids that cross the placenta are generally not oxidized but are stored in the liver and adipose tissue until birth. Upon birth, infants must fast until provided with maternal nourishment. During this period, liver glycogen stores are mobilized and are eventually depleted by 12 h after birth, both in fed and fasting infants. In addition, levels of fatty acids rise in the blood as triacylglycerol stores are mobilized. Milk, supplied by the mother, essentially provides a high-fat, low-

carbohydrate (lactose) diet. Infants remain on this high fat diet until weaning, when the diet shifts back towards a higher carbohydrate content.

Accompanying the changes in energy substrate metabolism immediately after birth, levels of insulin, which are elevated in the fetus, drop dramatically. Accordingly, glucagon levels, which were low in the fetus, rise abruptly in the post-natal period. The reason for the decline in insulin and rise in glucagon levels is unclear, although several hypotheses are review in Girard *et al.* (11).

Previous research in neonatal rabbit hearts, using an isolated working heart model, demonstrates that 1-day-old hearts are not able to function when perfused exclusively with fatty acids (12). In contrast, fatty acid oxidation appears to be mature in 7-day-old hearts, as isolated hearts could maintain function without consuming glycogen stores, when perfused with only fatty acids (12). There are several enzymes which, depending on changes in expression (level and/or isoform) and/or activity, could be responsible for the maturation of fatty acid oxidation. CPT 1, ACC, and AMPK have all been suggested as potential candidate enzymes (Figure I.8). The activity of CPT 1, in rabbit hearts, does not change from 1- to 7-days to 6-weeks after birth, whereas the activity of ACC decreases progressively over the same time period (13). Concomitant with the decrease in ACC activity is a decrease in malonyl CoA levels. In addition, hearts perfused in the absence of insulin experience a dramatic drop in ACC activity and malonyl CoA levels, and an increase in fatty acid oxidation rates. These data suggest that declining insulin levels may remove the malonyl CoA-induced

inhibition of CPT 1 and fatty acid oxidation. Furthermore, the expression of AMPK is greater in 7-day-old rabbit hearts than in 1-day-old rabbit hearts and the presence of insulin in the perfusate results in lower levels of AMPK activity (14). As AMPK phosphorylates and inhibits ACC, the presence of insulin most likely exerts its effects at the level of AMPK or higher. Thus, it appears that the decrease in circulating insulin upon birth results in increased AMPK activity, decreased ACC activity, lower levels of malonyl CoA, removal of CPT 1 inhibition and ultimately higher rates of fatty acid oxidation.

4) *Inotropes*

The concept of using inotropes to treat low cardiac output syndromes including chronic heart failure is centuries old. Cardiac glycosides have long been considered a suitable therapy for chronic heart failure since the efficacy of digitalis was recognized as effective in the treatment of "dropsy" (congestive heart failure). Cardiac glycosides act by inhibition of the Na^+/K^+ ATP-ase in the myocardium. Blockade of the Na^+/K^+ ATP-ase results in elevated intracellular Ca^{2+} levels via enhanced activity of the Na^+/Ca^{2+} exchanger [reviewed in Riaz *et al.* (15)]. Newer inotropes, known as phosphodiesterase inhibitors (i.e. amrinone and milrinone), have been studied for several decades with varying results. Drugs belonging to this class, primarily bipyridines, produce elevated levels of cAMP via inhibition of the enzymes (phosphodiesterases) that inactivate cAMP. Typically, these drugs, generally indicated for treatment of chronic heart failure, increase cardiac output

without an increase in heart rate. However, because of toxicity, they are indicated for acute therapy only.

While cardiac glycosides and phosphodiesterase inhibitors are effective in treating certain cardiac pathologies, infants with severe low cardiac output, due to anatomical malformations and/or surgery, are typically treated with adrenergic agonists (catecholamines).

Catecholamines evoke potent stimulation of two 2nd messenger systems via different subclasses of adrenergic receptors. Catecholamine stimulation of α adrenergic receptors (α-$AR_{1,2}$) activates the phospholipase C cascade leading to protein kinase C (PKC) activation and elevation of intracellular Ca^{2+} levels. Catecholamines also bind to and activate β adrenergic receptors (β-$AR_{1,2,3}$) leading to activation of adenylate cyclase and protein kinase A resulting in elevated intracellular Ca^{2+} levels. The ino- and chronotopic properties of the myocardium are primarily under the influence of β-$AR_{1,2}$, while in the vasculature, α-ARs increase vascular tone upon stimulation as opposed to β-ARs which induce relaxation of vascular smooth muscle. As catecholamines are administered systemically to infants, after and perhaps before surgery, it is preferable to use a non-specific adrenergic agonist. Agonists including epinephrine ($\beta = \alpha$), dopamine ($\beta > \alpha$) or dobutamine ($\beta > \alpha$), all of which stimulate β, and to some extent α–ARs, are the preferred approach. Isoproterenol is generally not preferable due to potent chronotropic stimulation, high risk of arrhythmias and

systemic vasodilation (Table I.1). Nevertheless, it is used in certain patients (those with bradycardia) and when attempting to elucidate molecular mechanisms, subtype specific agonists are preferable. The inotropic effects produced from the cAMP cascade are well understood, permitting a more transparent analysis of cellular events.

As mentioned previously, inotropes will stimulate ATP production in the myocardium directly (Ca^{2+} stimulation of PFK, PDC; cAMP stimulation of PFK) and indirectly (AMPK, PDC) via a decline in the cellular energy state (ATP, acetyl CoA). Research by Goodwin *et al.* (16,17) and Collins-Nakai *et al.* (18), demonstrate that the administration of an inotrope to isolated adult rat hearts results in a dramatic shift in percent energy contribution from fatty acid oxidation to glucose oxidation. Both groups have found that, while fatty acids provide the majority of energy for the myocardium before catecholamine administration, only carbohydrate metabolism increases to meet the increased energy demand. Goodwin *et al.* further suggest that the initial burst (5 min post-epinephrine treatment) in carbohydrate metabolism is supplied by glucose from glycogen stores (16). The authors state that glycogen is initially metabolized following inotrope administration while there is a delay in exogenous glucose metabolism limited at glucose uptake (17). After the initial burst in glucose metabolism and PDC activation, via dephosphorylation by PDC phosphatase, glucose oxidation from exogenous glucose provides the majority of ATP to meet the new inotrope-induced demand (17). Collins-Nakai *et al.* suggest the increase in glucose

metabolism, seen in their study, is consistent with previous work in which epinephrine stimulates glucose uptake, glucose phosphorylation, PFK and PDC (18). To explain the selective increase in glucose oxidation, without any increase in fatty acid oxidation, the authors suggest that PDC is the most sensitive dehydrogenase to epinephrine. In addition, the authors refer to unpublished data in which an increase in perfusate Ca^{2+} levels resulted in a selective increase in glucose oxidation as well.

5) *Congenital Heart Defects*

Generally, congenital heart disease is considered "a gross structural abnormality of the heart and intrathoracic great vessels that is actually or potentially of functional significance" [review in Hoffman (19)]. While this definition excludes particular abnormalities (i.e. persistent left superior vena cava and combined innominate-left carotid arterial trunk), the shear scarcity of these disorders precludes them from having an effect on overall CHD incidence.

The incidence of CHD in live-births is approximately 1% (Figure 9). The most prevalent malformations include ventricular septal defects (31%), atrial septal defects (7.5%), pulmonary stenosis (7%) and aortic coarctation (5.6%), with roughly 10 or so other identifiable cardiac defects comprising the remaining 49% [review in Hoffman (20)]. During the first half of the 20th century effective surgical correction was initiated for three specific defects [review in Higgins *et al.* (21). Repair for patent ductus arteriosus (1938), coarctation of the aorta (1944) and pulmonary valve stenosis (1948) were the first CHD to be surgically managed

adequately, resulting in survival into adulthood for many patients [review in Higgins *et al.* (21)]. As most CHD are genetic in origin, the incidence of these particular defects will continue to be prevalent, and ultimately lead to increased incidence of these particular defects, as more patients are reaching reproductive age [review in Hoffman (20)]. Nonetheless, at this interim, evidence suggests that the incidence of CHD has not significantly changed over the past 30 years, accounting for advances in CHD detection (ie. widespread use of echocardiography with color Doppler) [review in Hoffman (20)].

To treat CHD effectively, a multifaceted approach, which can be explained using the analogy of a series of concentric rings, is necessary. The outermost ring is peri-operative care. This includes adjunctive medication (i.e. inotropes, prostaglandin E_1). The next ring is the surgical intervention, from initial incision to wound closure. Finally, the innermost ring is myocardial protection during ischemia. Peri-operative care may involve administration of an inotrope pre-operatively, in severe conditions, and more commonly, post-operatively. Correction of the anatomical defect demands surgical expertise, however, contemporary correction of CHD relegates the myocardium to endure a period of myocardial ischemia, which necessitates effective cardioprotection. The use of inotropes in the peri-operative environment is to support cardiac output. However, treatment with inotropes is associated with a greater degree of low cardiac output post-ischemia (22).

6) *Ischemia and Myocardial Energy Substrate Metabolism*

The high energy demands of the myocardium necessitate that it is an obligate aerobe. Any deprivation of O_2 may be of serious consequence if not rectified in a timely manner. In conditions of severe ischemia ATP and glycogen are rapidly depleted, lactate accumulates, and eventually the heart will become necrotic [reviewed in Stanley *et al.* (8)]. Upon blood flow cessation, aerobic metabolism will generally cease as accumulation of acetyl CoA, NADH and $FADH_2$ inhibits glucose and fatty acid oxidation. In contrast, glycolysis will continue to metabolize glycogen until stores are depleted or until negative feedback from H^+ and NADH accumulation inhibits PFK and glyceraldehyde 3-phosphate, respectively.

Upon reperfusion, the myocardium is inefficient, as oxidative metabolism recovers quickly while recovery of mechanical function is much slower [reviewed in Stanley *et al.*(8)]. Return of pH to normal levels occurs rapidly, however, increases in intracellular Ca^{2+}, which may exacerbate injury, occur as well. Hydrogen extrusion and buffering during reperfusion occurs via several mechanisms including the lactate/H^+ co-transporter, Na^+/HCO_3^- co-transporter, Na^+/H^+ exchanger and vacuolar H^+-ATPase. The rapid activation of the Na^+/H^+ exchanger results in intracellular Na^+ build-up, Na^+ exchange via the Na^+/Ca^{2+} exchanger, and Ca^{2+} accumulation will occur (Figure I.9). High levels of intracellular Ca^{2+} can result in cell death [review in Opie (23)].

Studies, as reviewed by Stanley *et al.* (8), have shown that during reperfusion high rates of fatty acid oxidation and glycolysis occur, while glucose oxidation is suppressed. The presence of high plasma levels of fatty acids post-ischemia and low levels of malonyl CoA can suppress pyruvate oxidation, as mentioned previously. High levels plasma of fatty acids, and fatty acid oxidation, have been linked to depression of cardiac function during reperfusion. Several theories have addressed this observation, as reviewed by Lopaschuk *et al.* (5). One theory suggests that when rates of glycolysis greatly exceed glucose oxidation rates (uncoupling), excessive production of H^+ will occur. In the normal aerobic setting the myocardium can adequately manage H^+ production. Via the various H^+ transport and buffering mechanisms mentioned previously, and by virtue of H^+ incorporation into TCA cycle intermediates during TCA cycle flux, H^+ accumulation is of little concern. However, during ischemia and subsequent reperfusion, in which pyruvate oxidation is impaired, excessive H^+ production may result in increased intracellular Ca^{2+} accumulation.

7) *Rationale for Study*

Infants requiring surgery for correction of CHD must endure a period of myocardial ischemia. While many infants require inotropic support following surgery, the sickest will require inotropic therapy both before and after the procedure. As mentioned previously, treatment with inotropes is associated with a greater degree of low cardiac output during reperfusion.

Myocardial energy substrate metabolism can affect functional performance of the heart, particularly in the ischemia-reperfusion setting. Furthermore, catecholamines can have profound influence on myocardial metabolism. In adult rat studies, as mentioned previously, administration of an inotrope results in a shift of energy production from fatty acids to carbohydrates. In light of research showing that high rates of fatty acid oxidation are linked to depressed recovery during reperfusion, a shift of energy production from fatty acid oxidation to glucose oxidation may lessen this effect.

However, the newborn myocardium is in a state of maturation. How the newborn heart will respond to inotropic therapy in an aerobic period, in terms of metabolism, is unknown. The immature myocardium may shift metabolism as occurs in adults, or it may not. Therefore, my initial experimentation will be to determine the effects of an inotrope on energy substrate metabolism during an aerobic period with physiological levels of fatty acids (0.4 mM).

Clinically, it has been reported that infants given inotropes in the peri-ischemic setting tend to suffer post-operative low cardiac output. I hypothesize that the newborn heart may respond to inotropes, in terms of energy substrate metabolism, in such a manner that leads to an unfavorable recovery of function. If fatty acid oxidation does remain the major source of energy after treatment with an inotrope, I hypothesize that the newborn myocardium will have a depressed recovery from an ischemic insult if administered inotropes pre- or post-ischemia. I base this hypothesis on the evidence presented previously showing that high rates

of fatty acid oxidation will suppress rates of glucose oxidation resulting in an uncoupling of glycolysis from glucose oxidation. I further hypothesize that significant uncoupling of glycolysis from glucose oxidation pre-ischemia may enhance H^+ production during ischemia. Following isoproterenol stimulation, high rates of glycolysis before initiation of ischemia may result, once ischemia is initiated, in higher glycolytic rates than control hearts. I propose that increased glycogen breakdown during ischemia in isoproterenol-treated hearts will lead to more H^+ accumulation and upon reperfusion, higher levels of intracellular Ca^{2+}. In addition, I hypothesize that the administration of an inotrope upon reperfusion will promote uncoupling and exacerbate reperfusion injury greater than if an inotrope was not administered.

8) *Outline of Individual Studies*

Chapter III: examines the effects of isoproterenol on energy substrate metabolism in the aerobic setting.

Chapter IV: compares isoproterenol administration, before and after ischemia, on the metabolism of energy substrates in, and functional recovery of, neonatal hearts.

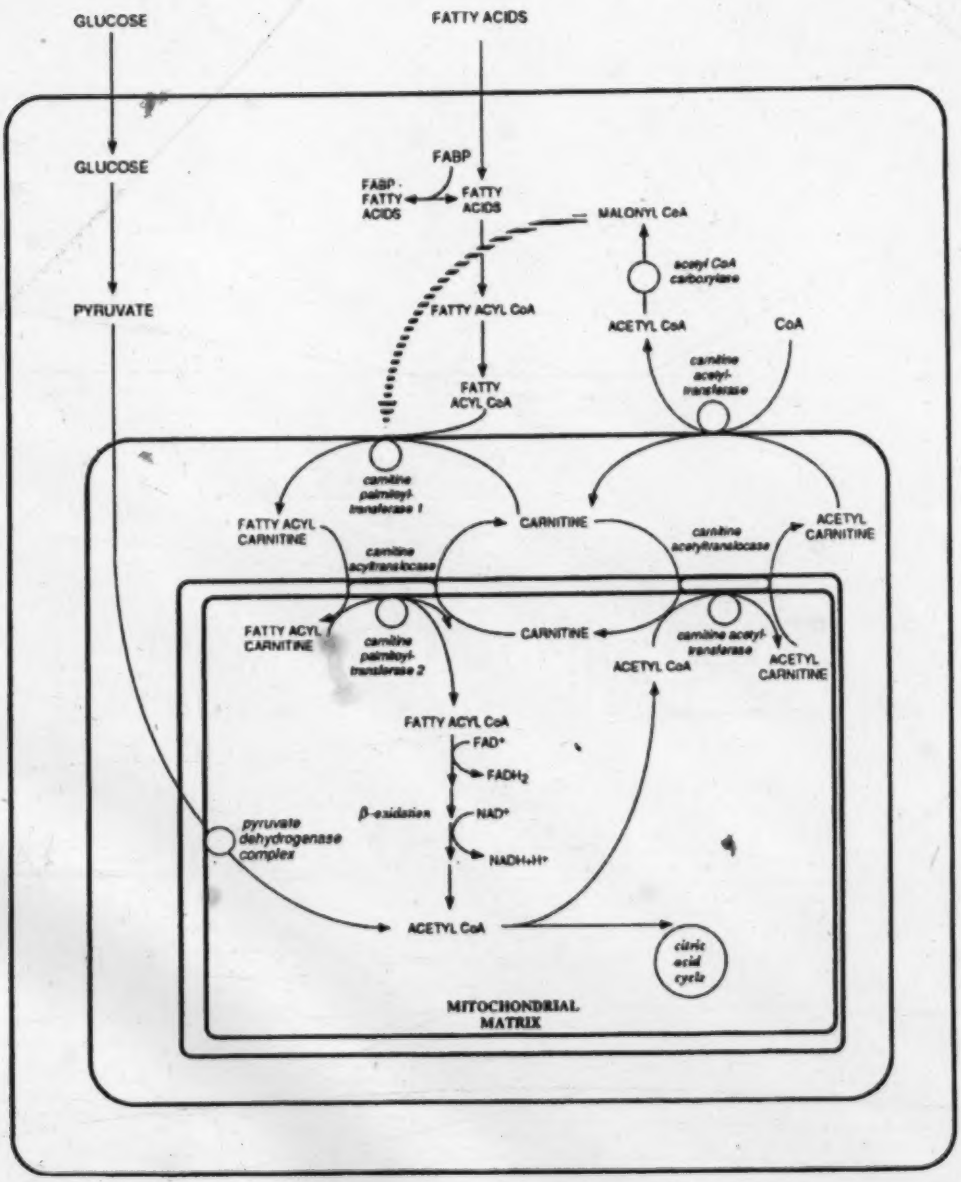

Figure I.1 General overview of cellular energy substrate metabolism. Lopaschuk GD, Belke DD, Gamble J, Itoi T, Schonekess BO 1994 Regulation of fatty acid oxidation in the mammalian heart in health and disease. Biochim Biophys Acta 1213:236-276

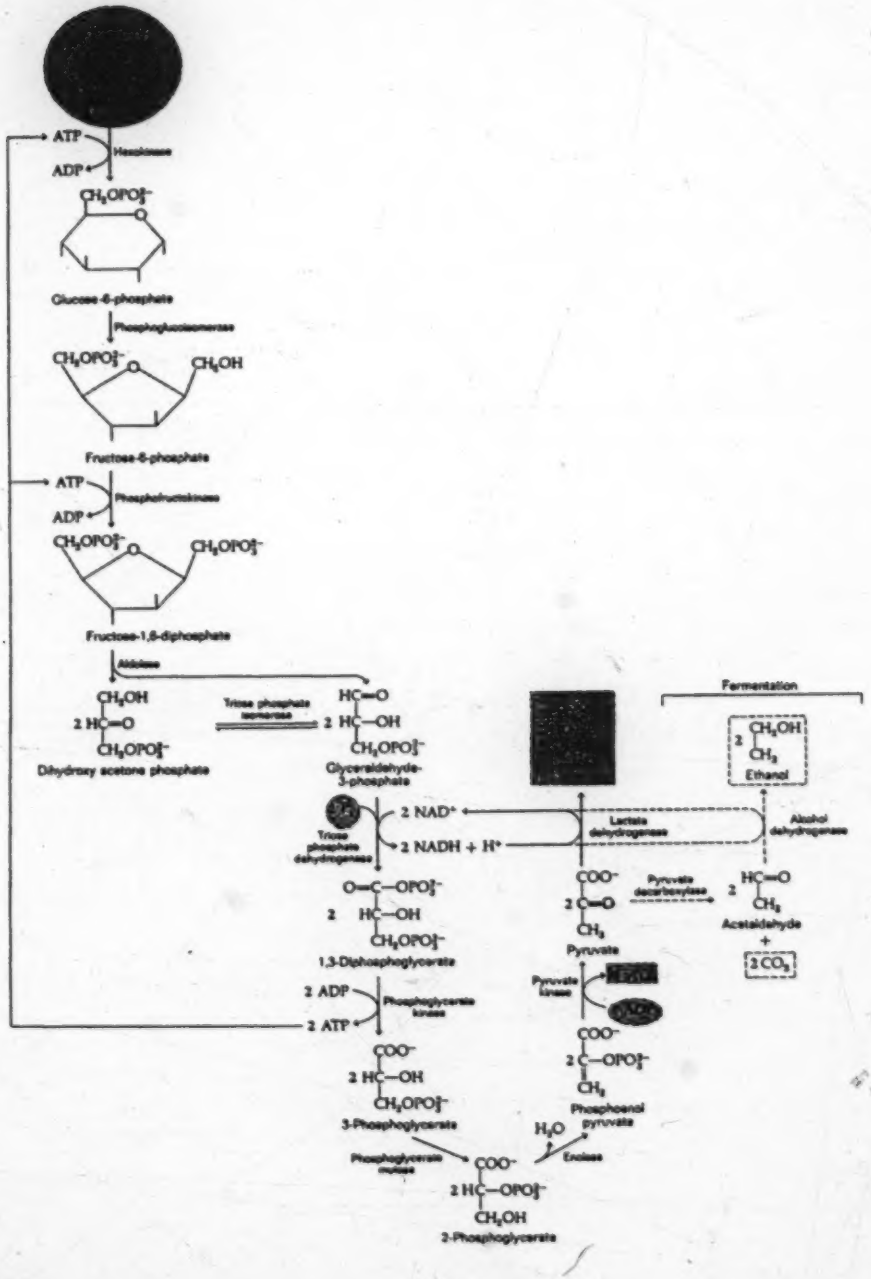

Figure I.2 Overview of glycolysis.
Papke GT & Anderson S *Introductory Biochemistry*. San Francisco: Holden-Day, 1973.

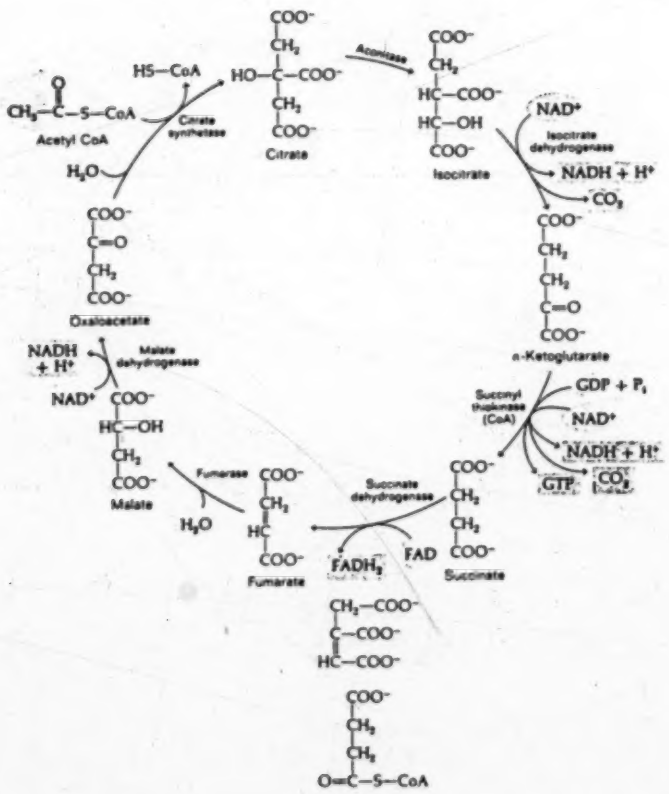

Figure I.3 Overview of glucose oxidation
Papke GT & Anderson S *Introductory Biochemistry*. San Francisco: Holden-Day, 1973.

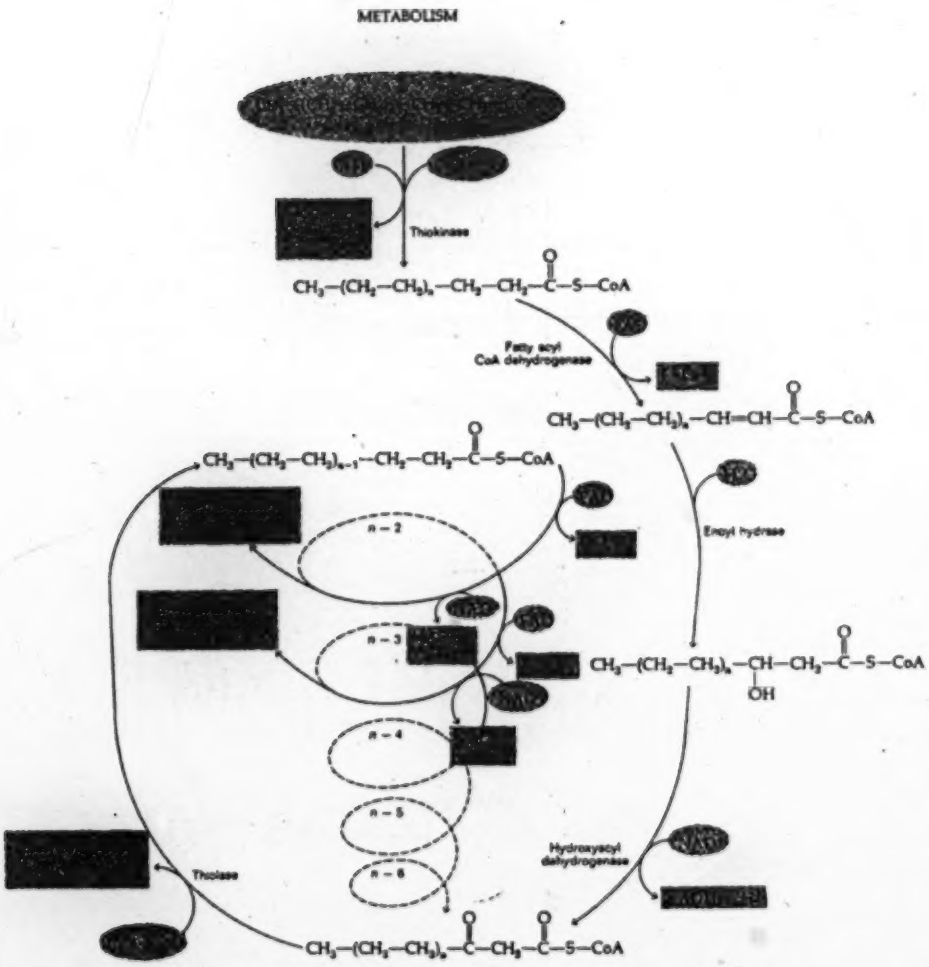

Figure I.4 Fatty acid oxidation
Papke GT & Anderson S *Introductory Biochemistry*. San Francisco: Holden-Day, 1973.

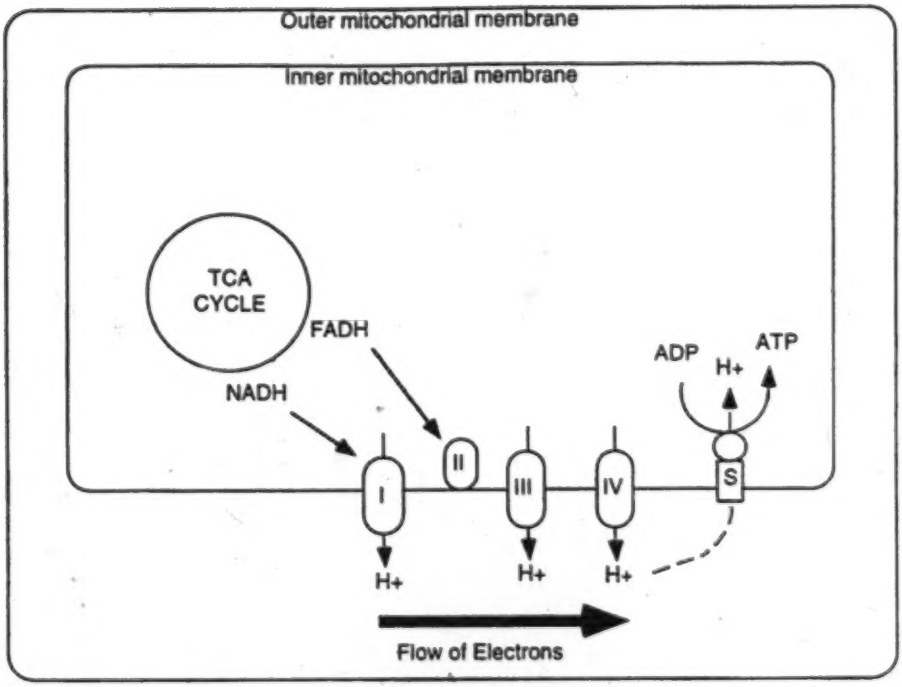

Figure I.5 Electron transport chain (ETC). I, ETC complex I; II, ETC complex II; III, ETC complex III; IV, ETC complex IV; S, ATP synthase. Complex I receives electrons from NADH, while complex II receives electrons from FADH. Belke DD 1997 Hypothermia and heart metabolism.

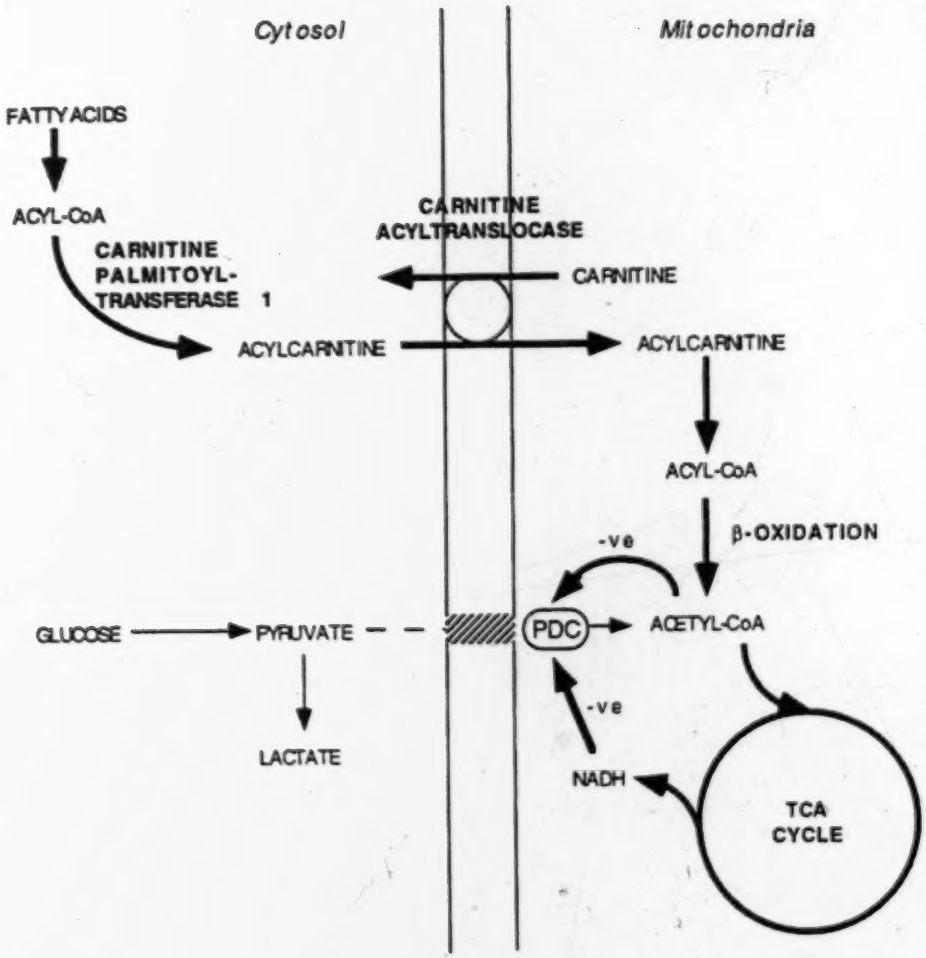

Figure I.6 Inhibition of glucose oxidation by fatty acid oxidation.
Belke DD 1997 Hypothermia and heart metabolism

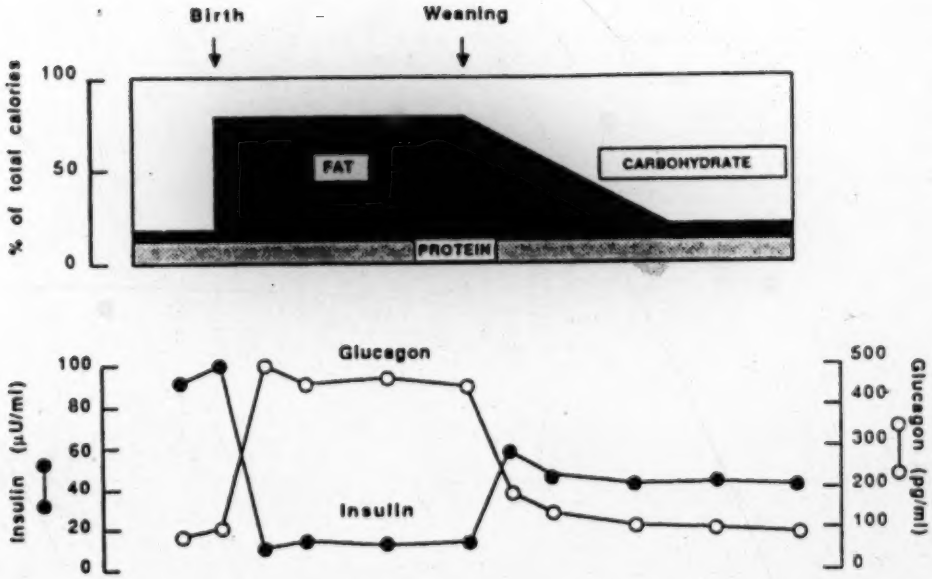

Figure I.7 Changes in diet and levels of selected hormone in the peri-natal period. Girard J, Ferre P, Pegorier JP, Duee PH 1992 Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. Physiological Reviews 72:507-562

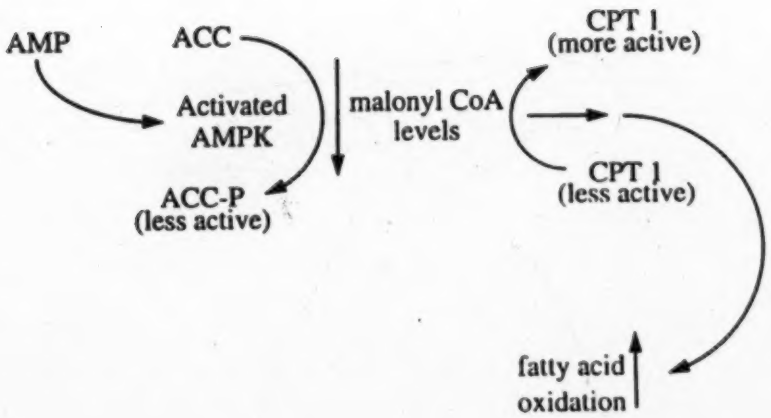

Figure I.8 Interactions of AMPK, ACC and CPT 1.
Lopaschuk GD 1997 Alterations in fatty acid oxidation during reperfusion of the heart after myocardial ischemia. Am J Cardiol 80(3A):11A-16A

	HR	*PAP*	*PCWP*	*MAP*	*CI*	*SVR*	*MvO_2*
Epinephrine	↑	↑	↑	↑	↑	⇅	↑
Isoproterenol	⇈	↓	↓	↓	↑	↓	↑
Dopamine	↑	↑	↑	↑	↑	⇅	↑
Dobutamine	↑	↑→	↓	↓→	↑	↓	↑→
Calcium		↑	↑	↑	↑	↑	↑
Amrinone		↓→	↓	↓→	↑	↓	↓

Abbreviations: HR, heart rate; PAP, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; MAP, mean arterial pressure; CI, cardiac index; SVR, systemic vascular resistance; MvO_2, myocardial oxygen consumption.

Table I.1 Overview of the hemodynamic effects of various inotropes.
DiSesa VJ 1991 Pharmacological support for postoperative low cardiac output. Seminars in Thoracic and Cardiovascular Surgery 3:'3-23

Years of birth	Place of study	Total live births	Total congenital heart disease	Congenital heart disease/ 1000 live births
1941–1950	Gothenburg, Sweden [12]	58,105	363 (388)	6.25 (6.68)[a]
1946–1953	New York City [75]	5,628	43	7.64
1951–1960	Gothenberg, Sweden [13]	58,314	450	7.72
1952–1961	Uppsala, Sweden [65]	48,500[b]	291	6.00
1958	Leiden, Netherlands [46]	1,817	15	8.25
1950–1969	Olmsted County, Minnesota [26]	32,393	186	5.74
1959–1967	USA multicenter [66]	54,765	420	7.67
1957–1971	Blackpool, England [8]	56,982	338	5.95
1959–1966	Northern California [41]	19,044	163 (199)[c]	8.56 (10.45)[c]
1960–1969	Liverpool & Bootle, England [23]	160,480	884	5.51
1963–1973	Denmark [51]	854,886	5,249	6.14
1963–1965	Budapest, Hungary [22]	52,569	373	7.10
1963–1965	Szolnok County, Hungary [64]	5,644	67 (74)[c]	11.87 (13.11)[c]
1955–1969	Pécs, Baranya, Hungary [73]	97,482	744	7.63
1975	Switzerland [86]	78,464	494	6.30
1980	Bohemia, Czechoslovakia [80]	91,823	589	6.41
1981	Sweden [14]	94,778	853 (1010)[c]	9.00 (10.66)[c]
1971–1984	Dallas County, TX [31]	379,561	2,509	6.61 (7.55)[d]
1975–1984	Florence, Italy [60]	46,895	579	12.35
1976–1980	Czechoslovakia [17]	203,000	1,279	6.30
1976–85	Oviedo, Spain [24]	53,578	279	5.21
1979–83	Tyrol, Austria [30]	41,725	341	8.17
1979–86	Bas-Rhin, France [88]	105,374	801	7.60
1981–1982	Baltimore-Washington [28]	368,889	1,498	4.05
1981–1984	Alberta, Canada [33]	103,411	573	5.54[e]
1981–1987	Bratislava, Czechoslovakia [47]	61,420	480	7.82
1982–1988	Vestfold Co., Norway [63]	15,307	138	9.02
1986–1987	Hainaut, Belgium [7]	17,647	132	7.48
1988–1990	Guadeloupe, FWI [16]	22,855	139	6.08

[a] Including patients detected after the initial report.
[b] Estimated.
[c] Including possible heart disease as well.
[d] Estimated after allowing for underestimation of ventricular septal defects in earlier years.
[e] During the first year of life only.

Table I.2 Incidence of congenital heart disease in live-birth infants.
Hoffman JJE 1995 Incidence of congenital heart disease: I. Postnatal Incidence. Pediatric Cardiology 16:103-113

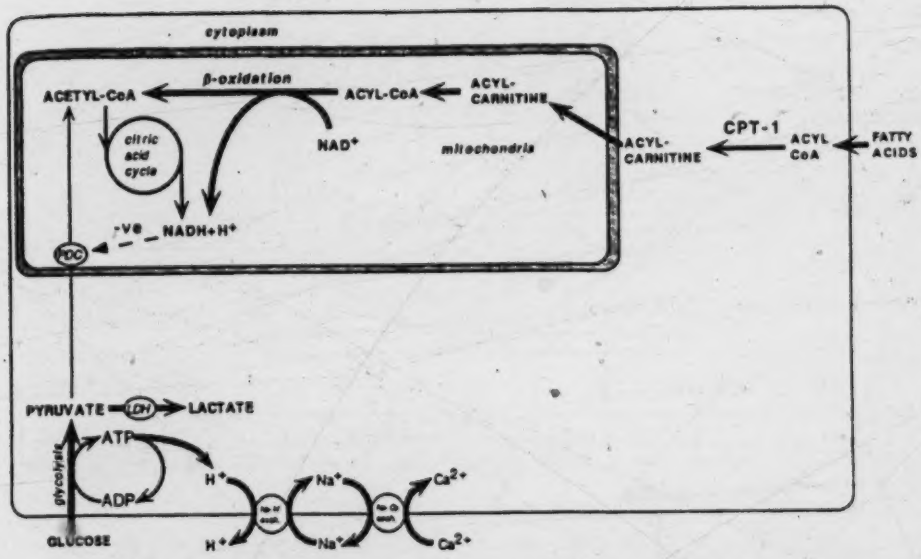

Figure I.9 H^+ production, as a result of uncoupling of glycolysis from pyruvate oxidation and its contribution to Ca^{2+} overload.
Belke DD 1997 Hypothermia and heart metabolism.

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12. Lopaschuk GD, Spafford MA 1990 Energy substrate utilization by isolated working hearts from newborn rabbits. Am J Physiol 258:1274-1280

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Chapter II

Materials and Methods

II.1) *Materials*

Isoproterenol was obtained from the Sigma Chemical Company. Radiolabeled substrates ([U-^{14}C]-glucose, [5-^{3}H]-glucose, [9,10-^{3}H]-palmitate and [U-^{14}C]-lactate) were purchased from New England Nuclear. Hyamine® hydroxide (methylbenzethonium; 1 M in methanol) was purchased from ICN Radiochemicals. Bovine serum albumin (fraction 5) and acetyl CoA were obtained from Boehringer Mannheim. The Alberta Peptide Institute synthesized the AMARA (AMARASAAALARRR) peptide used in the AMPK assay. AG® 1-X4 exchange resin was from BioRad Laboratories, and Scintisafe® 30% aqueous counting scintillant was purchased from Fisher Scientific. All other chemicals were reagent grade.

II.2) *Isolated Heart Perfusions*

Hearts were obtained from 7-day-old New Zealand White rabbits (Vandemeer Farms, Edmonton, Alberta). All animals were cared for according to the guidelines of the Canadian Council on Animal Care. On the morning of experimentation, hearts were excised following sodium-pentobarbital anesthetization (60 mg • kg^{-1} i.p.). The aorta was quickly cannulated, and a 60 mm Hg 37°C retrograde perfusion with Krebs'-Henseleit solution (pH 7.4, gassed with 95% O_2/5% CO_2) containing 2.5 mM $CaCl_2$ and 11 mM glucose was initiated

as previously described (1- 3). During this initial perfusion, hearts were trimmed of excess tissue and the pulmonary artery and left atrium were each cannulated. Hearts were then switched to the working mode, in a closed recirculating system for $^{14}CO_2$ collection (1-3), and perfused at a 7.5 mm Hg left atrial filling pressure and a 30 mm Hg hydrostatic aortic afterload. The perfusate (100 ml) contained 11 mM glucose, 3% albumin, 2.5 mM $CaCl_2$, either 0.4 or 1.2 mM palmitate, 0.5 mM lactate and 100 $\mu U \bullet ml^{-1}$ insulin. A concentration of 11 mM glucose was chosen in order to saturate glucose uptake and maintain tissue glycogen levels. The perfusion protocol consisted of A) a 40 min aerobic period, or 2) a 30 min aerobic period, 30 min global no-flow ischemic period and a 40 min aerobic reperfusion period (Figure II.1). Isoproterenol (dissolved in ascorbic acid), when added, was administered at 5 min (Iso 0 min) or 60 min (Iso 60 min) into the 100 min perfusion. The concentration of isoproterenol used ($3 \bullet 10^{-7}$ M) was chosen since preliminary experiments showed that it produced the maximum increase in cardiac work in 7-day-old rabbit hearts. Heart rate, peak systolic pressure and developed pressure were monitored throughout with a Harvard Apparatus® 60-3002 pressure transducer in the aortic line. Transonic® flow probes placed in the aortic outflow and left atrial preload line (cardiac output). All signals were recorded with an Acq*knowledge*® 3.2.5 computerized transduction system for the MP100WSW from Biopac Systems. Cardiac work was determined as the product of peak systolic pressure and cardiac output.

II.3) *Measurements of glycolysis, glucose oxidation, lactate oxidation and fatty acid oxidation*

Glycolysis and glucose oxidation were measured simultaneously by perfusing hearts with perfusate containing tracer [5-^{3}H/U-^{14}C]-glucose, as described previously (1-3). Lactate oxidation and palmitate oxidation were measured in separate but parallel experiments using tracer [9,10-^{3}H]-palmitate and [U-^{14}C]-lactate (2,4).

Rates of glycolysis were determined by measuring $^{3}H_2O$ production, released at the enolase step of glycolysis (Figure II.2), after its separation from radiolabeled glucose using AG® 1-X4 columns, as described previously (1-3). Rates of either glucose or lactate oxidation were determined by measurement of $^{14}CO_2$ production in the closed perfusion system that allowed collection of both gaseous and perfusate $^{14}CO_2$. Buffer and gaseous $^{14}CO_2$ production were measured at 10 min intervals throughout the perfusion period. The $^{14}CO_2$ is released from [U-^{14}C]-glucose or [U-^{14}C]-lactate at the level of PDC and at the level of the TCA cycle (Figure II.2) (2,4).

Palmitate oxidation rates were obtained by measuring perfusate $^{3}H_2O$ production (Figure II.3) from [9,10-^{3}H]-palmitate after its separation from [9,10-^{3}H]-palmitate using a chloroform/methanol extraction, as described previously (2). All rates are expressed as either nanomoles or micromoles of substrate used per min per gram of dry weight of tissue and were linear over the time periods

measured. At the end of the perfusions, hearts were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2 and were stored at -80°C until further biochemical analysis or determination of dry-to-wet weight ratio (1).

II.4) *Enzyme Activity*

II.4.1) *Pyruvate Dehydrogenase Complex (PDC) Assay*

PDC analysis was performed using a modification of the radioisotopic coupled enzyme assay developed by Constantin-Teodosiu *et al.* (5). Frozen ventricular tissue samples (30 - 40 mg) were homogenized for 30 s in the presence of 360 - 370 µl of 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 50 mM NaF, 5 mM dichloroacetate (DCA) and 0.1% Triton X-100 (pH 7.8). To measure total PDC (PDC_t) activity, NaF and DCA were omitted from the homogenization buffer, and 10 mM glucose and 2.25 U hexokinase • ml^{-1} buffer were added to the buffer. The homogenate was then incubated for 15 min at 37°C in the presence of 200 mM sucrose, 4.8 mM $CaCl_2$, 12 mM $MgCl_2$, 2.5 mM DCA, 50 mM KCl, 0.5 mM EGTA, 100 mM glucose, 2.25 U hexokinase and 50 mM Tris-HCl. Following this, 100 µl of homogenate was added to 480 µl of assay buffer containing 150 mM Tris-HCl (pH 7.8), 0.75 mM NAD^+, 0.75 mM CoA and 1.5 mM thiamine pyrophosphate. The reaction was initiated by the addition of pyruvate (30 µl of a 26 mM stock

solution) to a final concentration of 1 mM. After 10 min, the reaction was terminated by the addition of 210 µl of 0.5 M perchloric acid. The solution was then neutralized, centrifuged, and the supernatant acetyl CoA content measured. Activity was based on the measurement of acetyl CoA in which acetyl CoA formed from the PDC assay (active (PDC_a) or total) was converted to [^{14}C]-citrate in the presence of citrate synthase and [^{14}C]-oxaloacetate. Sodium glutamate and aspartate aminotransferase were used to remove excess [^{14}C]-oxaloacetate after the citrate synthase reaction by transamination of unreacted oxaloacetate to [^{14}C]-aspartate. AG® 50W-X8 resin (100-200 mesh) was then used to separate [^{14}C]-aspartate from [^{14}C]-citrate. Acetyl CoA content of supernatant samples was quantified by comparison with acetyl CoA standard curves run in each experiment.

II.4.2) *Extraction of 5'-Adenosine Monophosphate Activated Protein Kinase (AMPK) and Acetyl CoA Carboxylase (ACC)*

Approximately 200 mg of frozen powdered tissue was homogenized with a buffer containing 50 mM Tris-HCl (pH 7.0 at 4°C), 250 mM mannitol, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM ethylene-diaminetetra-acetic acid (EDTA), 1 mM EGTA, 1 mM dithiothretiol, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 4 µg • ml^{-1} soybean trypsin inhibitor, and 1 mM benzamidine. Samples were then centrifuged at 14,000g for 20 min at 4°C. The supernatant was then brought to 2.5% polyethylene glycol (PEG) with 25% (wt/vol) PEG 8000 and agitated for 5 min at 4° C. The supernatant was then made

up to 6% PEG 8000 using PEG 8000 stock described above and stirred once again for 5 min at 4° C. This fraction was then spun at 10,000g for 10 min, and the precipitate was washed with homogenization buffer containing 6% PEG 8000. This was followed by a final centrifugation at 10,000g, after which the protein concentration in the supernatant was measured using a Sigma BC® protein kit.

II.4.3) *5'-AMP-activated Protein Kinase Assay*

AMPK activity was measured according to the method described by Hardie's group (6-8), with slight modifications (9-11). Briefly, 2 µl of the PEG fraction was added to a reaction mixture (final volume, 25 µl) composed of 40 mM HEPES-NaOH, 80 mM NaCl, 8% (wt/vol) glycerol, 0.8 mM EDTA, 200 µM [^{32}P]-ATP, 5 mM $MgCl_2$, and 0.18% Triton X-100. Samples were incubated in the presence or absence of 200 µM AMP at 30°C for 5 min. This mixture was then incubated for 3 min at 30°C. From this incubation mixture, 15 µl was spotted on 1 cm^2 phosphocellulose paper. The paper was then washed four times for 10 min each with 150 mM phosphoric acid, followed by a 5 min acetone wash. Papers were then dried and counted for radioactivity. AMPK activity was expressed as picomoles ^{32}P incorporated into the AMARA peptide per min per mg protein.

II.4.4) *Acetyl CoA Carboxylase Assay*

ACC activity in the PEG fractions was measured using the CO_2 fixation method (12). The reaction mixture (final volume, 165 µl) containing 60.6 mM Tris acetate, 1 mg • ml^{-1} bovine serum albumin, 1.32 µM β-mercaptoethanol, 2.2

mM ATP, 1.06 mM acetyl CoA, 5.0 mM magnesium acetate and 18.08 mM $NaH^{14}CO_3$ was pre-incubated with 10 mM citrate at 37°C. Following 2 min of incubation 5 µl of the PEG fraction, containing 20 µg of the total protein, was added. Samples were incubated at 37°C for 2 min, and the reaction was stopped by adding 25 µl of 10% perchloric acid. Samples were then spun for 20 min at 3500 rpm, and 160 µl of supernatant was placed in minivials and dried in a fume hood overnight. H_2O (100 µl), followed by scintillant, was then added to the vials, and the vials were counted. ACC activity was expressed as the nanomoles of malonyl CoA produced per min per mg protein.

II.5) Tissue Metabolites

II.5.1) *Determination of CoA Esters*

CoA esters were obtained from powdered tissue as described previously (11). The 6% perchloric acid extract was maintained at a pH of 2 - 3. The CoA esters were measured using a modified high performance liquid chromatography (HPLC) procedure described by King *et al.* (13). Separation was performed using a Beckman System Gold® with UV detector 167. Each sample (100 µl each) was run through a pre-column cartridge (C18, size 3 cm, 7 µm) and a Beckman Ultrasphere® column (type C18, particle size 3 µm, size 4.6 • 100 mm). Absorbance was set at 254 nm and flow rate of 1 ml • min^{-1}. A gradient was initiated using two buffers: buffer A consisted of 0.2 M NaH_2PO_4 (pH 5) and buffer B was a mixture of 0.25 M NaH_2PO_4 (pH 5) and acetonitrile in a ratio of

80:20 (v/v). Buffers were filtered using filter pure, Nylon-66® filter membrane from Pierce. Initial conditions (97% A and 3% B) were maintained for 2.5 min and were changed thereafter to 18% B over 5 min using Beckman's® curve 3. At 15 min the gradient was changed linearly to 37% B over 3 min and subsequently to 90% B over 17 min. At 42 min the composition was returned linearly back to 3% B over 0.5 min, and at 50 min column equilibration was complete. Peaks were integrated by a Beckman System Gold® software package.

II.5.2) *Extraction of Nucleotides and Lactate*

Nucleotides and lactate were extracted from frozen ventricular tissue (100 ± 10 mg). Tissue was homogenized on ice in 1.0 ml 6% PCA/0.5 mM EGTA. Homogenate was centrifuged at 11,000 rpm for 2 min and 95 µl of 0.32 M DTT was added to the supernatant. Samples were neutralized with 5 M K_2CO_3 to pH 6.8 ± 0.5, centrifuged at 11,000 rpm for 2 min, and the supernatant was frozen immediately in liquid nitrogen until later assessment of nucleotides and lactate.

II.5.3) *Nucleotides*

For determination of nucleotides, 100 µl of supernatant were analyzed by HPLC, as previously described (15). Nucleotides, AMP degradation products, PCr and Cr were resolved with 35 mM K_2HPO_4, 6 mM tetrabutyl-ammonium hydrogen sulfate buffer, pH 6.0, and a binary acetonitrile gradient on medium-bore, 250 • 3.9 mm steel octadecyl-bonded (C18) columns at a flow rate of 1.5 or 1.0 ml • min^{-1}. Peaks were integrated by a Beckman System Gold® software package.

II.5.4) *Lactate*

A portion of the supernatant (II.5.2) was used to measure lactate via enzymatic reaction with lactate dehydrogenase and glutamate pyruvate transaminase as previously described (16,17). Assay buffer (60 ml) containing 0.54 M glycylglycine and 0.11 M glutamic acid (pH to 10.0), was added in 0.5 ml aliquots to each tube followed by 0.45 ml of H_2O, 0.1 ml of 50 mM NAD^+ and 10 µl of 10 mg • ml^{-1} glutamate pyruvate transaminase. After addition of 50 µl of extracted sample, a initial measure of optical density at 340 nm was taken. Following addition of 10 µl of 5 mg • ml^{-1} lactate dehydrogenase, samples were incubated for 30 min and optical density was reexamined.

II.6) *Statistics*

All data are presented as the mean ± SE. The unpaired Students *t* test, Welch's *t* test, Mann-Whitney Rank Sum test, one-way ANOVA followed by Bonferroni post-hoc comparison or one-way ANOVA on ranks (Kruskal-Wallis) followed by a Dunn's post-hoc comparison were used to determine the statistical difference where appropriate. A value of $P < 0.5$ was considered significant.

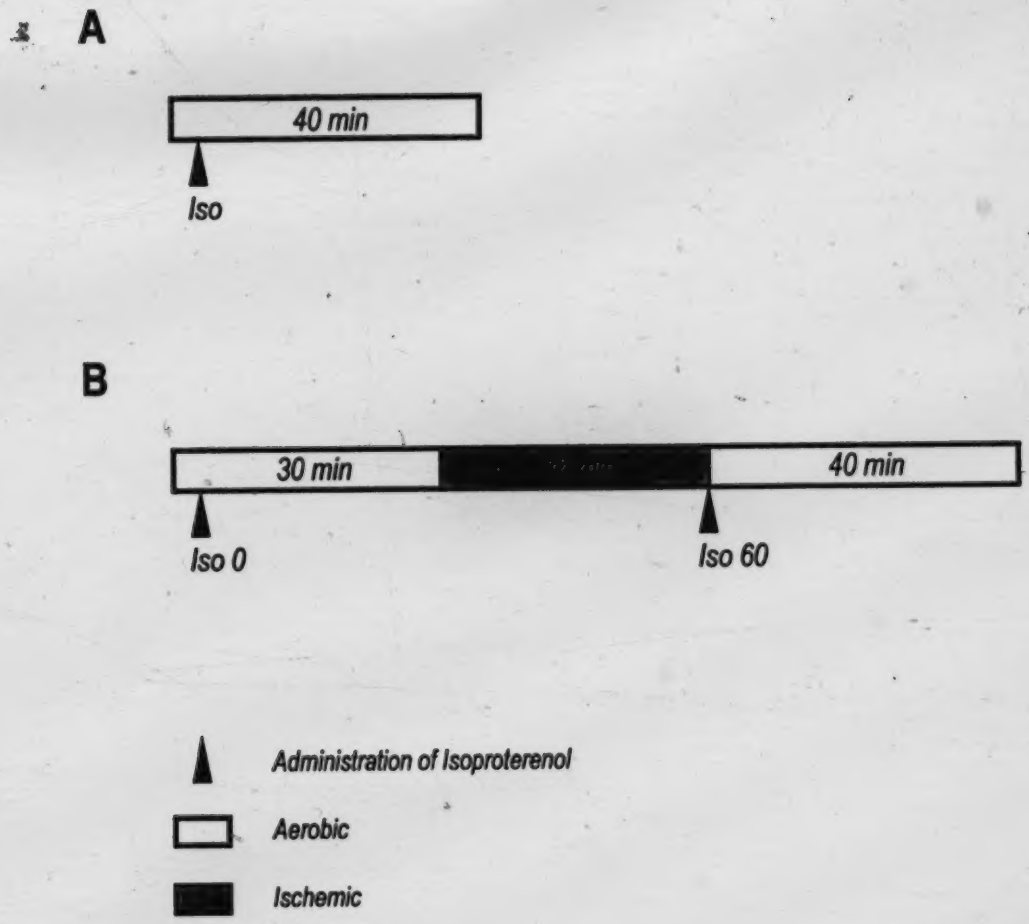

Figure II.1 Perfusion protocols.
A) 11 mM glucose, 3% albumin, 2.5 mM $CaCl_2$, 0.5 mM lactate, 0.4 mM palmitate, 100 μU • ml-1 insulin; B) 11 mM glucose, 3% albumin, 2.5 mM $CaCl_2$, 0.5 mM lactate, 1.2 mM palmitate, 100 μU • ml-1 insulin

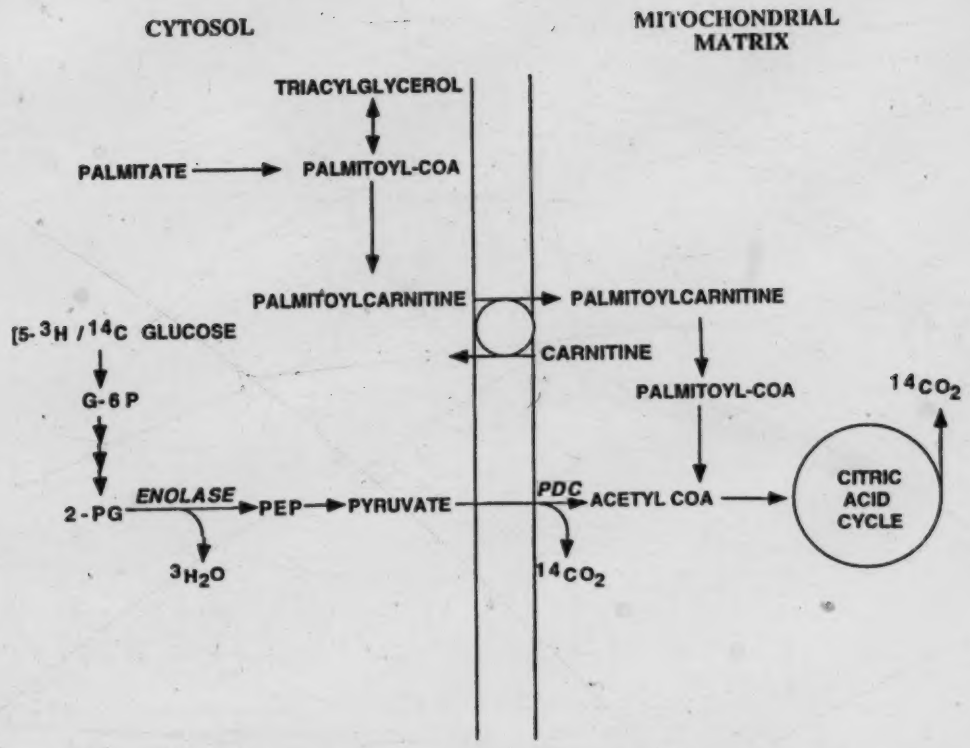

Figure II.2 Production of $^{3}H_20$ and $^{14}CO_2$ from the metabolism of glucose and lactate. Belke DD 1997 Hypothermia and Heart Metabolism.

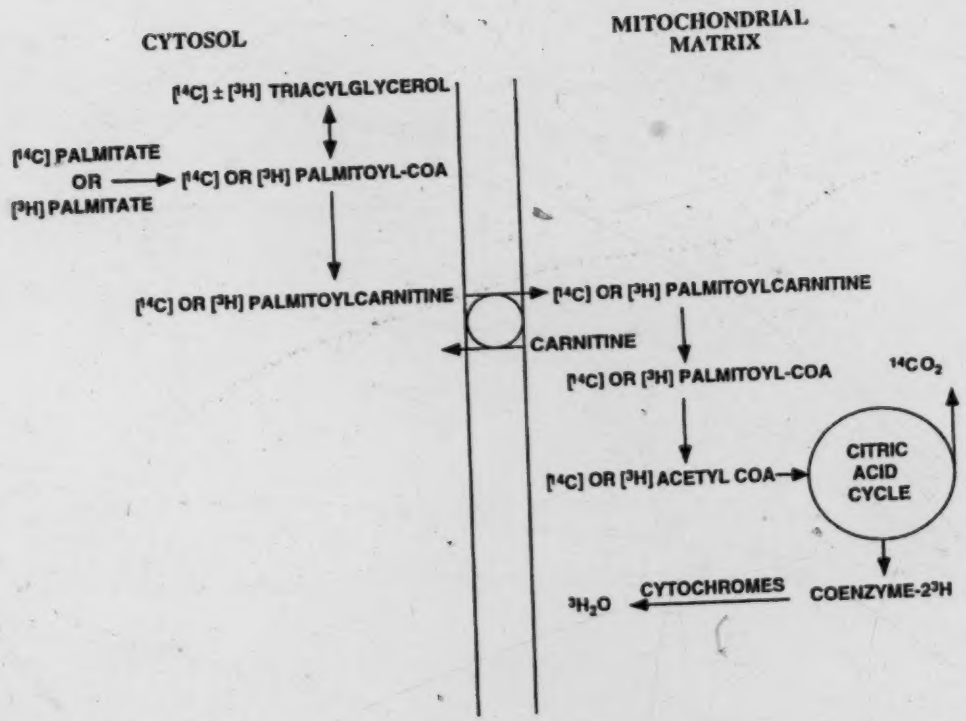

Figure II.3 Production of $^{3}H_20$ or $^{14}CO_2$ from the oxidation of palmitate.
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Chapter III

Fatty Acid Oxidation Remains the Preferred Energy Source of the Newborn Rabbit Heart Following Isoproterenol Treatment

Introduction

Energy substrate preference in the heart undergoes rapid changes during the early newborn period. As mentioned previously, by 7-days of age, ATP contribution from glycolysis dramatically decreases and the contribution from palmitate oxidation greatly increases, becoming the primary source of energy for the heart (1,2). The post-natal increase in fatty acid oxidation rates is due, in part, to a decrease in malonyl CoA levels (3). The decrease in malonyl CoA levels following birth occurs secondary to an increase in 5'-AMP-activated protein kinase (AMPK) (4) activity which phosphorylates and inhibits acetyl CoA carboxylase (ACC) (3), the enzyme responsible for cytoplasmic malonyl CoA production.

During the transformation from carbohydrate to fatty acid metabolism in the newborn heart, glucose oxidation remains very low (1,2). PDC is the rate-limiting step in the oxidation of carbohydrates. In the adult rat heart, PDC activity dramatically increases following catecholamine treatment (5). This increase in PDC activity by inotropic agents is secondary to an increase in intramitochondrial calcium concentrations [reviewed in Stanley *et al.* (6)]. Whether catecholamines increase carbohydrate metabolism in the newborn heart is not known.

Although a rapid and dramatic change in myocardial energy substrate metabolism occurs in the newborn heart, it is unclear how an increase in work affects metabolism and energy substrate preference. As mentioned previously, epinephrine treatment in adult rats caused a dramatic rise in glucose oxidation with no change in fatty acid oxidation, resulting in a shift in energy preference from fatty acid oxidation to glucose metabolism (5,7,8).

The type of energy substrates used in the ischemic setting (such as cardiac surgery) can affect cardiac performance (9,10). As a result, since metabolic responses (activity of AMPK, ACC and PDC) to inotropic stimulation in the newborn are unknown it is important to understand what effect catecholamines have on energy substrate preference. The objective of this study was to determine what effect isoproterenol has on glycolysis, glucose oxidation, lactate oxidation and fatty acid oxidation in the newborn heart. These studies were performed in isolated working hearts obtained from 7-day-old rabbits since it has been previously observed that the maturation of fatty acid oxidation has already occurred in these hearts. In addition, the influence of isoproterenol on levels of CoA esters and key regulatory enzymes of glucose oxidation (PDC) and fatty acid oxidation (AMPK and ACC) were assessed.

Materials and Methods

Materials

Refer to Chapter II section 1.

Isolated Heart Perfusions

Methods are as presented in Chapter II.2, with the following amendments. The perfusate contained 0.4 mM palmitate. The perfusion protocol consisted of a 40 min working heart aerobic perfusion. Isoproterenol, when present, was administered at 5 min into the working heart perfusion.

Measurements of Glycolysis, Glucose Oxidation, Lactate Oxidation and Fatty Acid Oxidation

As described in Chapter II.3.

Pyruvate Dehydrogenase Complex Assay

As described in Chapter II.4.1.

Determination of CoA Esters

As described in Chapter II.5.1.

Extraction of AMPK and ACC

As described in Chapter II.4.2.

5'-AMP-activated Protein Kinase Assay

As described in Chapter II.4.3.

Acetyl CoA Carboxylase Assay

As described in Chapter II.4.4.

Statistics

As described in Chapter II.6.

Results

Effects of Isoproterenol on Mechanical Function

The addition of $3 \bullet 10^{-7}$ M isoproterenol significantly increased most measured parameters of cardiac function (Table III.1). A significant increase in heart rate, cardiac output, aortic flow and cardiac work was seen in isoproterenol-treated hearts, compared to control hearts. No significant changes were seen in peak systolic pressure.

Effects of Isoproterenol on Glycolysis, Glucose Oxidation, Lactate Oxidation and Palmitate Oxidation

Cumulative rates of glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation throughout the 40 min perfusion period are shown in Figures III.1 through III.4, respectively. Addition of isoproterenol produced a significant linear increase in rates of glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation. Steady state rates of glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation are shown in Figures III.5 through III.8, respectively. Glycolysis increased 296%, glucose oxidation 282%, lactate oxidation 202%, and palmitate oxidation 139%.

Tricarboxylic Cycle Activity

TCA cycle activity (Figure III.9) is measured as the contribution of acetyl CoA from the catabolism of each particular substrate (glucose, palmitate, lactate)

to the TCA cycle. In control hearts, fatty acid oxidation contributed 82% of the acetyl CoA for the TCA cycle, while overall carbohydrate oxidation (glucose and lactate) provided only 19%. In isoproterenol-treated hearts, the contribution of acetyl CoA from glucose oxidation increased 282%, lactate oxidation 202%, and palmitate oxidation 139%. Despite the greater increase in carbohydrate oxidation, fatty acid oxidation remained the primary source of TCA cycle acetyl CoA (75%).

Relative Calculated ATP Production

Figure III.10 shows the contribution of glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation to relative rates of ATP production in hearts perfused in the presence or absence of isoproterenol. There was an increase in the contribution of ATP from glycolysis (17% to 23%) and glucose oxidation (10% to 14%). Lactate oxidation contribution remained constant at 7% and palmitate oxidation contributed relatively less ATP (67% to 56%) after isoproterenol treatment.

PDC Activity Following Isoproterenol Treatment

The effects of isoproterenol treatment on PDC activity are presented in Figures III.11 and III.12. The activity of PDC (Figure III.11) increased significantly in isoproterenol treated hearts (86%). Consequently, the percent PDC_a to PDC_t increased following isoproterenol treatment from 36% active to 60% active (Figure III.12).

Effects of Isoproterenol on CoA Esters

The levels of CoA esters in the absence or presence of isoproterenol are presented in Table III.2. The levels of acetyl CoA and malonyl CoA decreased significantly by 46% and 88%, respectively. Levels of free CoA virtually did not change where as acetyl CoA/free CoA ($p = 0.064$) and succinyl CoA ($p = 0.066$) levels trended downward but failed to reach significance.

Effects of Isoproterenol on ACC and AMPK Activity

AMPK activity in the absence or presence of isoproterenol is presented in Figure III.13. In either the absence or presence of AMP (200 μM), isoproterenol significantly increased AMPK activity by 32% and 35%, respectively.

The activity of ACC in the absence or presence of isoproterenol is presented in Table III.3. Isoproterenol treatment resulted in a non-significant 16% decrease in ACC activity.

Table III.1

Isoproterenol Effects on Mechanical Function in Isolated Working Hearts from 7-Day Old Rabbits

Parameter	Control (n = 16)	Isoproterenol (n = 15)
Heart Rate (beats•min^{-1})	241 ± 7	348 ± 9*
Peak Systolic Pressure (mm Hg)	50 ± 1	51 ± 1
Cardiac Output (ml•min^{-1})	25 ± 2	36 ± 3*
Aortic Flow (ml•min^{-1})	18 ± 1	29 ± 2*
Cardiac Work (ml•min^{-1}•mm Hg•10^{-2})	13 ± 1	18 ± 1*

Data are mean values of measurements obtained at 10, 20, 30 and 40 min after initiation of the working heart perfusion. Values are the mean ± SE.

*Significantly different from control hearts.

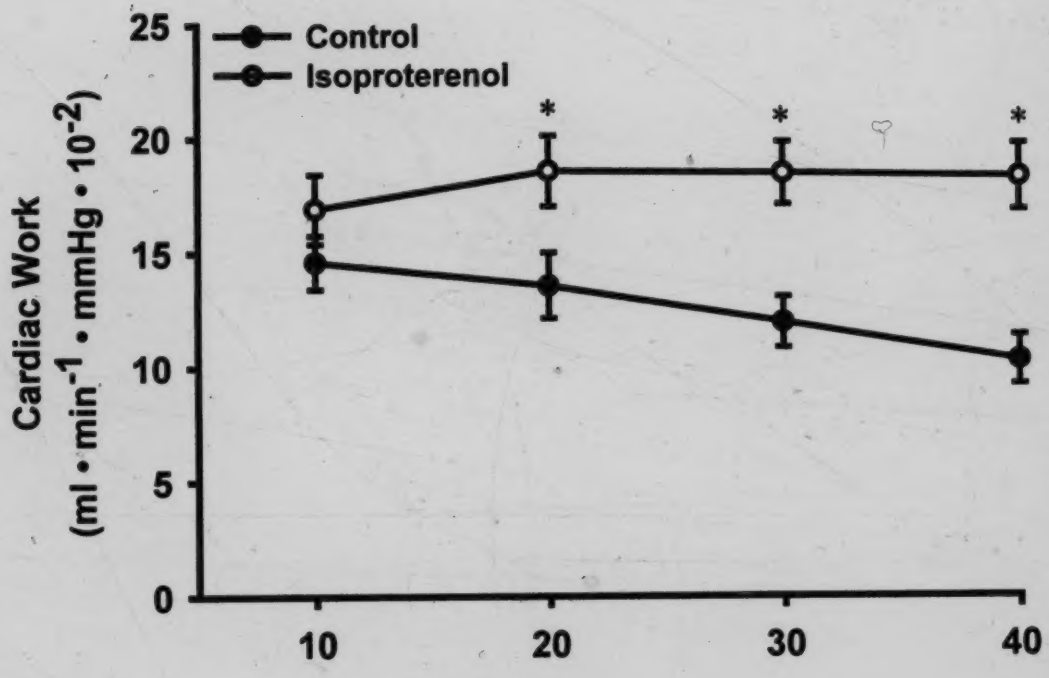

Figure III.1 Effects of isoproterenol on the time course of cardiac work in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=16; Isoproterenol, N=15.

* Significantly different from control hearts at the corresponding perfusion time.

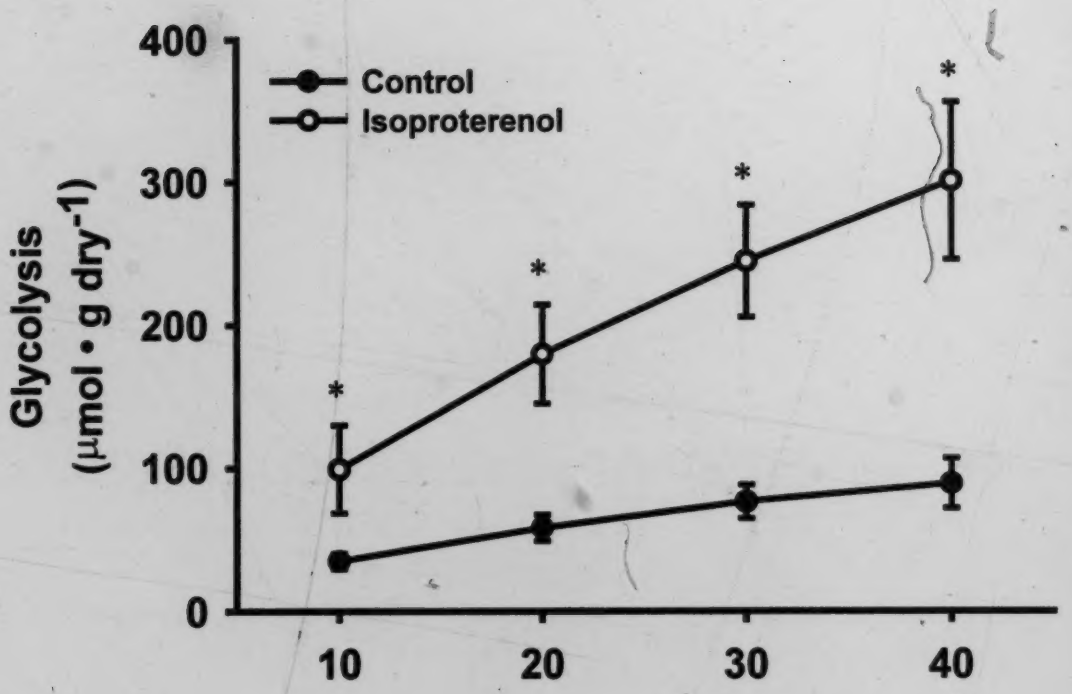

Figure III.2 Effects of isoproterenol on the time course of glycolysis in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=9; Isoproterenol, N=8.

* Significantly different from control hearts at the corresponding perfusion time.

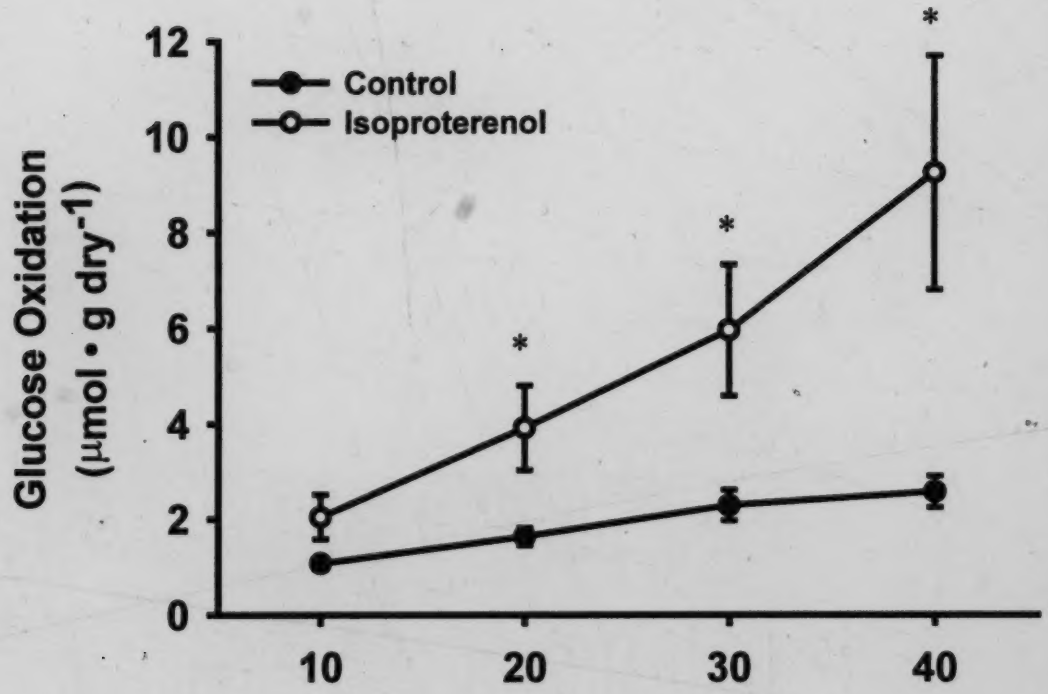

Figure III.3 Effects of isoproterenol on the time course of glucose oxidation in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=9; Isoproterenol, N=9.

* Significantly different from control hearts at the corresponding perfusion time.

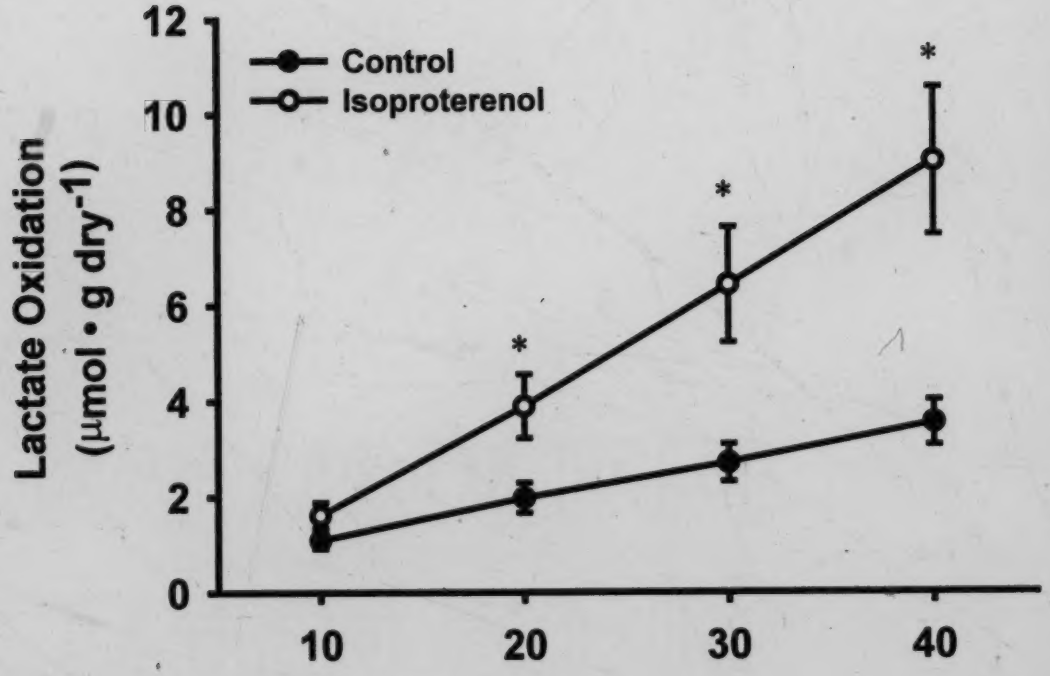

Figure III.4 Effects of isoproterenol on the time course of lactate oxidation in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=8; Isoproterenol, N=6.

* Significantly different from control hearts at the corresponding perfusion time.

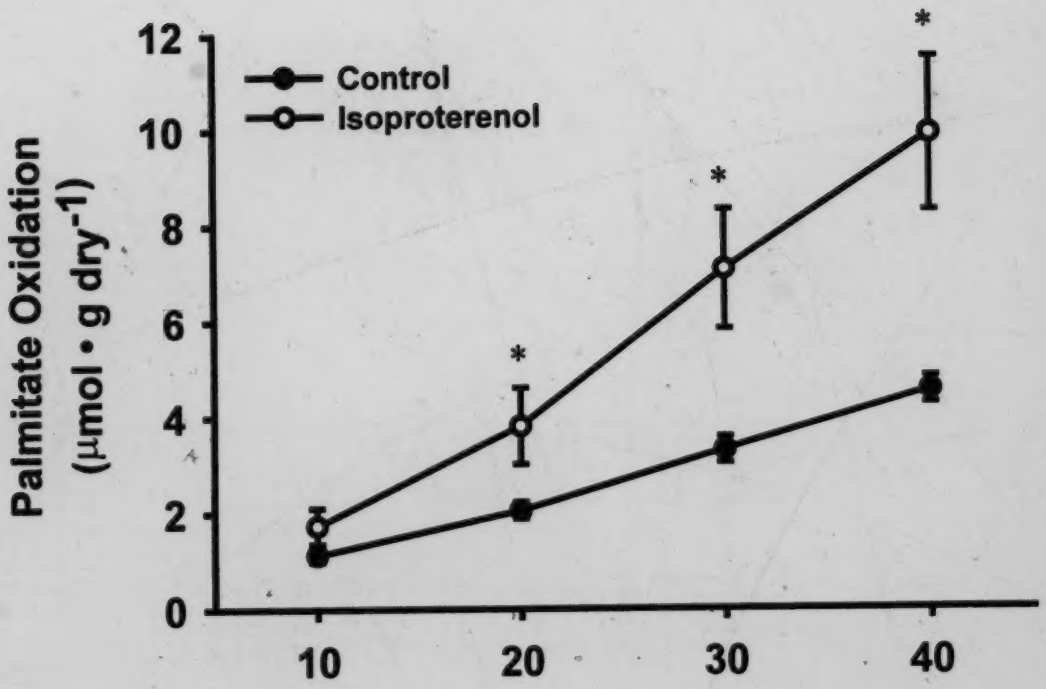

Figure III.5 Effects of isoproterenol on the time course of palmitate oxidation in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=8; Isoproterenol, N=6.

* Significantly different from control hearts at the corresponding perfusion time.

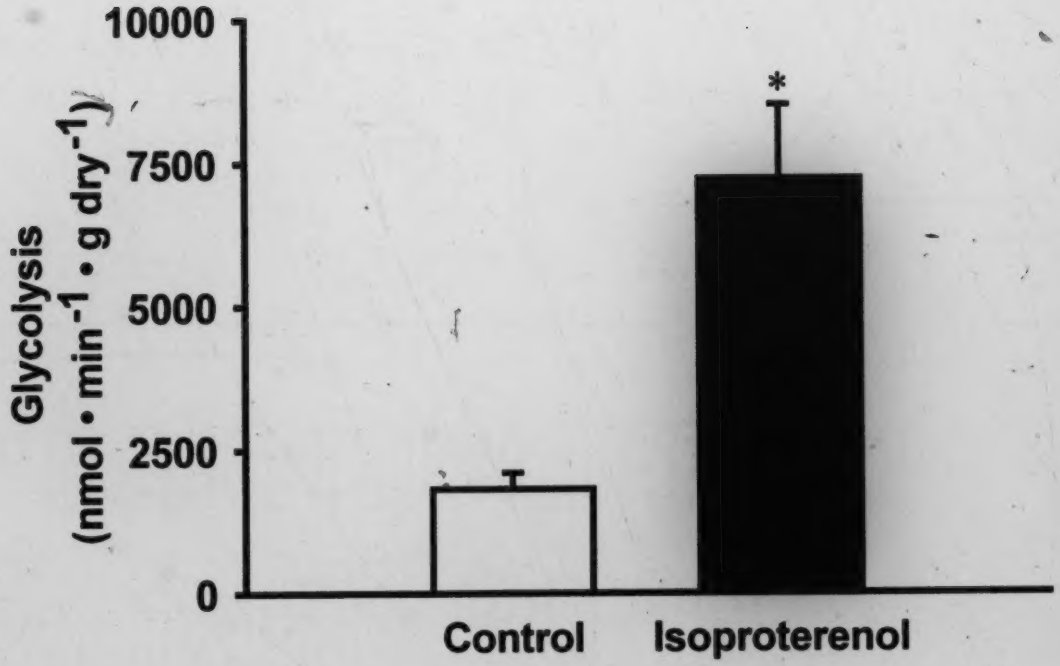

Figure III.6 Effects of isoproterenol on the steady state rates of glycolysis in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 and 40 min after initiation of working heart perfusion. Values are the mean ± SE. Control, N=9; Isoproterenol, N=8.
* Significantly different from control hearts.

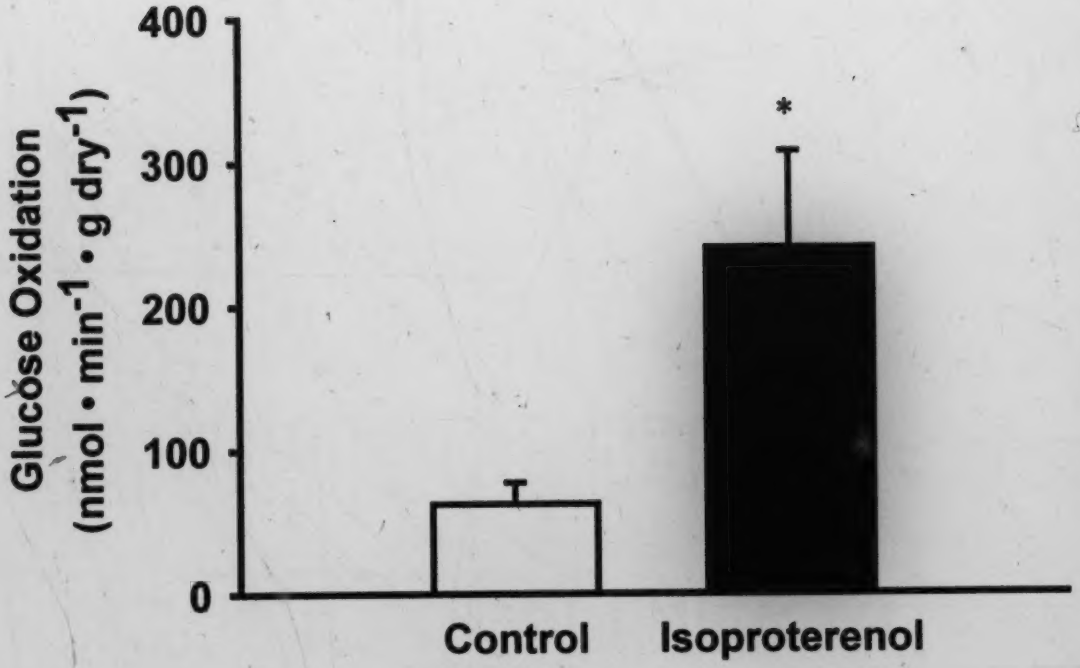

Figure III.7 Effects of isoproterenol on the steady state rates of glucose oxidation in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 and 40 min after initiation of working heart perfusion. Values are the mean ± SE. Control, N=9; Isoproterenol, N=9.
* Significantly different from control hearts.

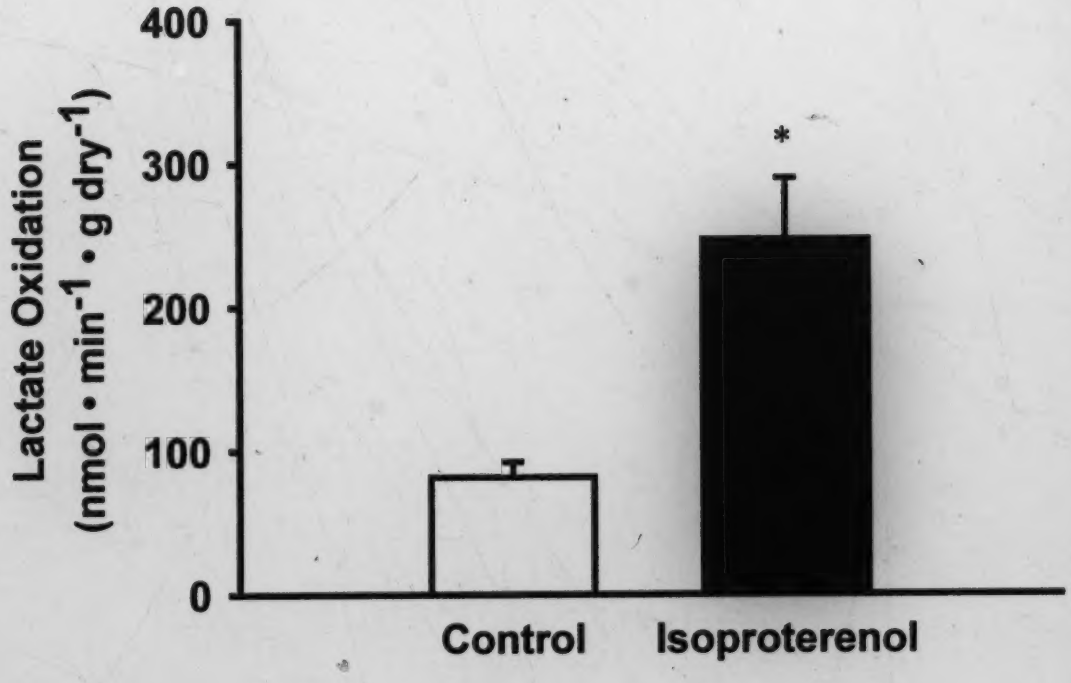

Figure III.8 Effects of isoproterenol on the steady state rates of lactate oxidation in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 and 40 min after initiation of working heart perfusion. Values are the mean ± SE. Control, N=8; Isoproterenol, N=6.
* Significantly different from control hearts.

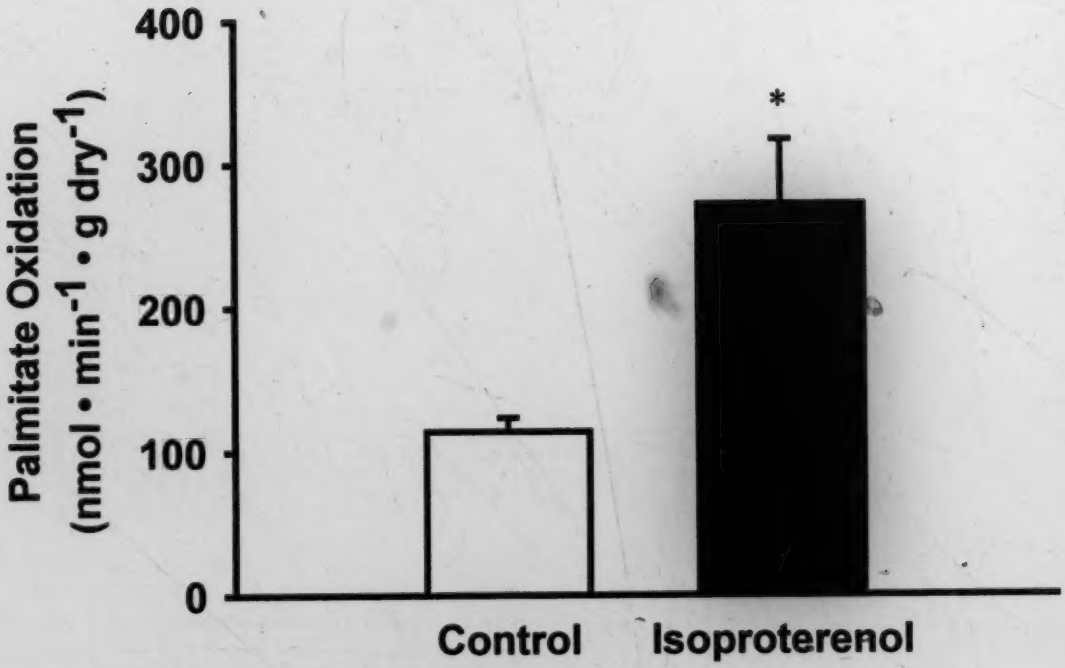

Figure III.9 Effects of isoproterenol on the steady state rates of palmitate oxidation in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 and 40 min after initiation of working heart perfusion. Values are the mean ± SE. Control, N=8; isoproterenol, N=6.
* Significantly different from control hearts.

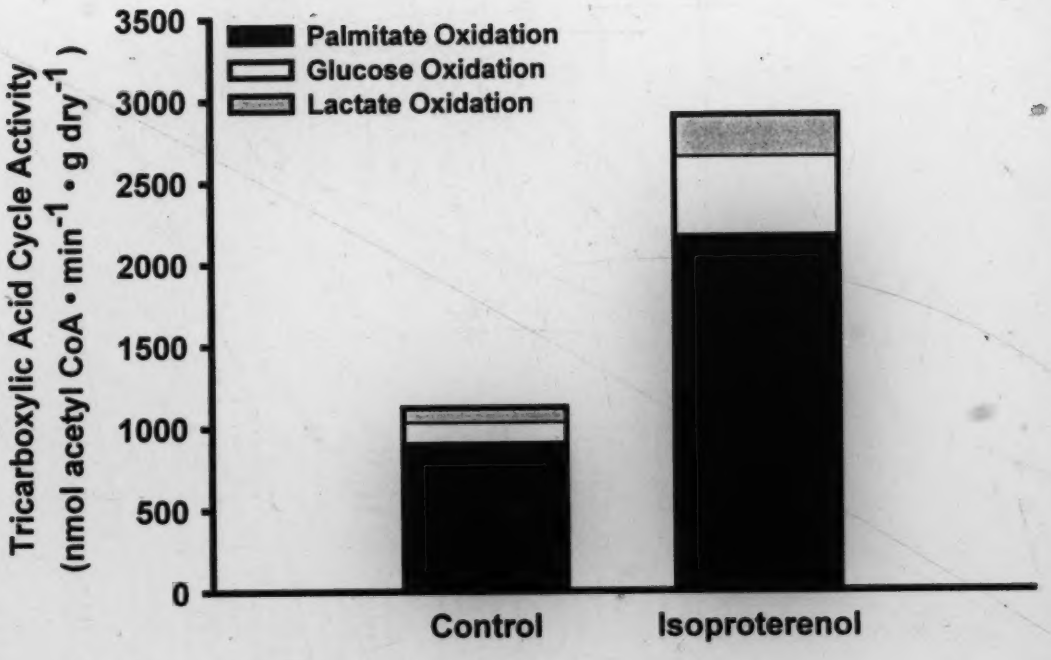

Figure III.10 Effects of isoproterenol on absolute rates of acetyl CoA production from glucose oxidation, lactate oxidation and palmitate oxidation in isolated working 7-day-old rabbit hearts.

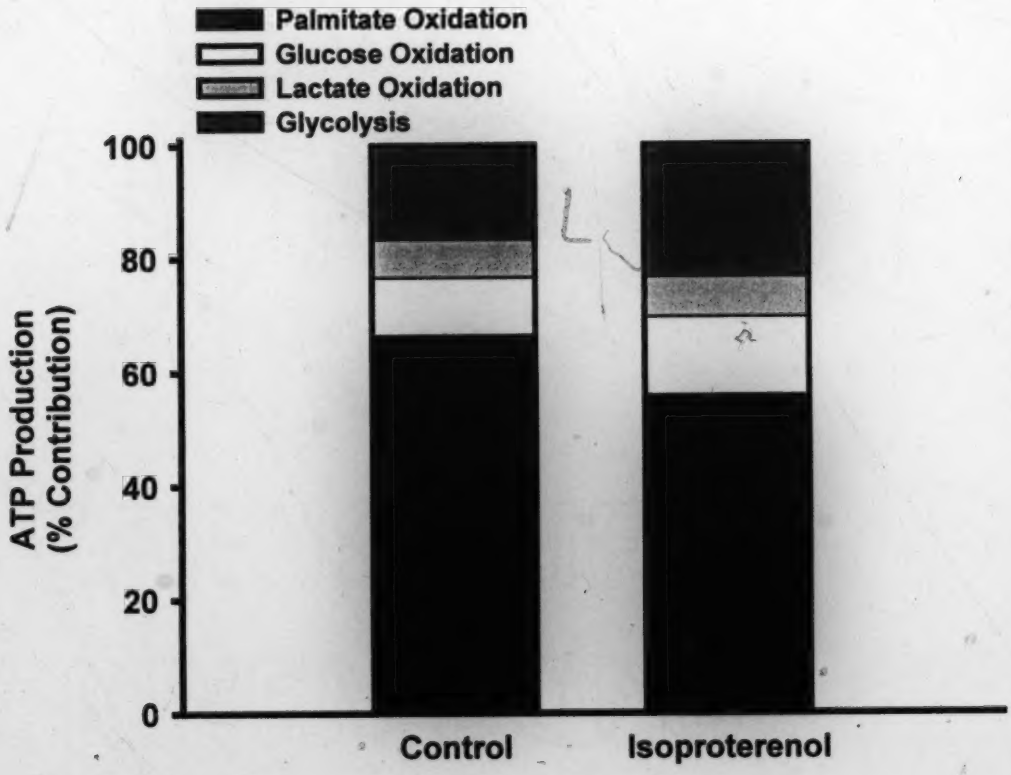

Figure III.11 Effects of isoproterenol on relative rates of ATP production from glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation in isolated working 7-day-old rabbit hearts.

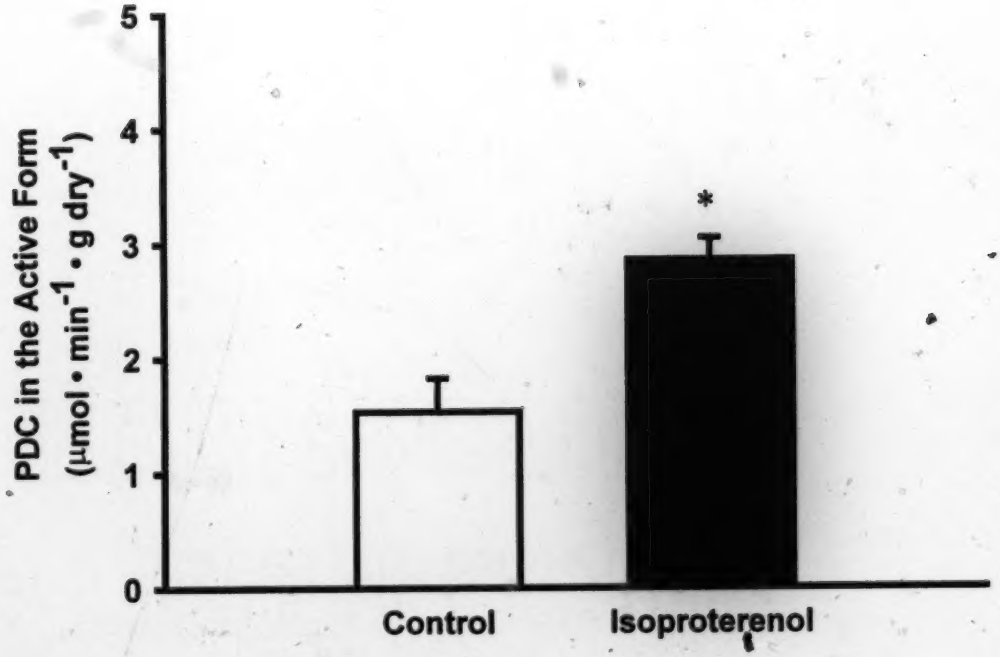

Figure III.12 Effects of isoproterenol on PDC in the active form in isolated working 7-day-old rabbit hearts. Data are measurements of values obtained at 40 min after the initiation of working heart perfusion. Values are the mean ± SE. Control, N=8; isoproterenol, N =8.

* Significantly different from control hearts.

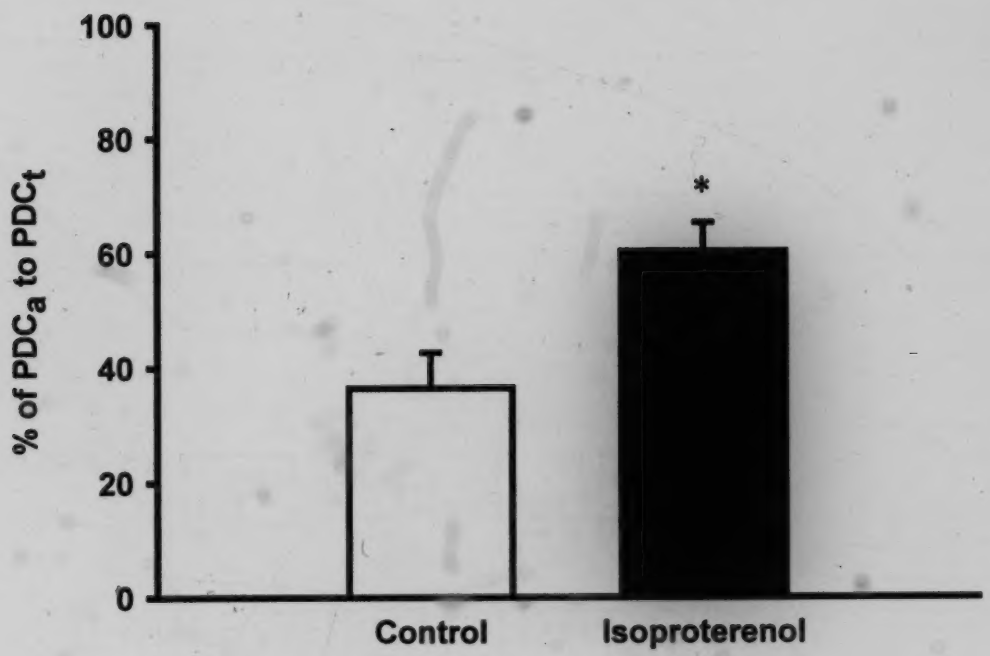

Figure III.13 Effects of isoproterenol on percent of active PDC to total PDC in isolated working 7-day-old rabbit hearts. Data are measurements of values obtained at 40 min after the initiation of working heart perfusion. Values are the mean ± SE. Control, N =8; isoproterenol, N=8.

* Significantly different from control hearts

Table III.2

Isoproterenol Effects on CoA Esters in Isolated Working Hearts from 7-Day-Old Rabbits

CoA Ester	Control (N = 8)	Isoproterenol (N = 8)
	(nmol • g dry^{-1})	
Free CoA	89.0 ± 7.7	86.4 ± 9.2
Acetyl CoA	23.1 ± 4.1	12.6 ± 1.1*
Acetyl CoA / CoA	0.26 ± 0.05	0.15 ± 0.02
Malonyl CoA	7.5 ± 1.3	0.9 ± 0.9*
Succinyl CoA	22.9 ± 2.7	14.6 ± 3.3

Data are mean values of measurements obtained after 40 min of working heart perfusion. Values are the mean ± SE.

*Significantly different from control hearts.

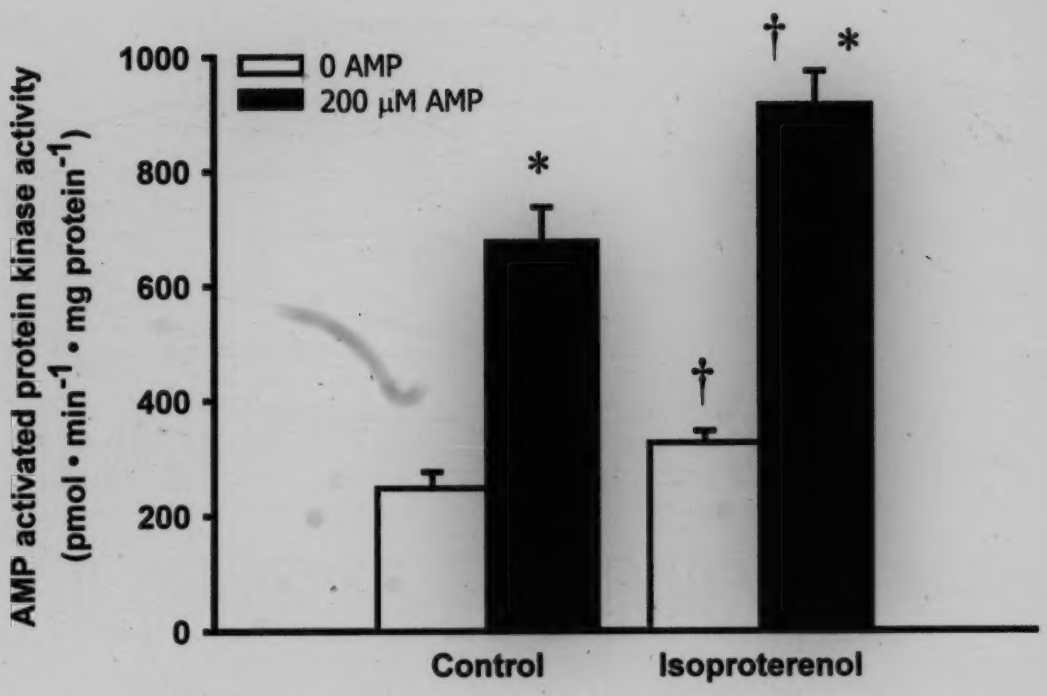

Figure III.14 Effects of isoproterenol on AMPK activity in isolated working 7-day-old rabbit hearts. Data are measurements of values obtained at 40 min after the initiation of working heart perfusion. Values are the mean ± SE. Control, N=16; Isoproterenol, N=15.

* Significantly different from 0 AMP hearts.

† Significantly different from control hearts.

Table III.3

Isoproterenol Effects on ACC Activity in Isolated Working Hearts from 7-Day-Old Rabbits

	Control (N = 16)	Isoproterenol (N = 15)
	(nmol • min^{-1} • mg $protein^{-1}$)	
Acetyl CoA Carboxylase	2.58 ± 0.19	2.23 ± 0.13

Data are mean values of measurements obtained after 40 min of working heart perfusion. Values are the mean ± SE.

Discussion

This study was designed to determine the relative changes in energy substrate preference in the newborn heart after treatment with an inotrope. The results show that there are little relative changes, resulting in fatty acid oxidation remaining the major source of energy for the heart after treatment with isoproterenol. These results contrast experiments in adult models where treatment with inotropes results in a shift of energy supply from fatty acid oxidation to glucose oxidation (5,7,8).

Accompanying the increase in fatty acid oxidation rates produced by isoproterenol was increased activity of AMPK. This increase in AMPK activity can result in inactivation of ACC following phosphorylation (11). As inactivation of ACC will result in lower levels of malonyl CoA, as seen in this study, there will be less inhibition of CPT 1 and fatty acid oxidation will increase.

However, ACC activity was already low in 7-day-old hearts and was not significantly depressed by this increase in AMPK activity. Since the activity of ACC is also dependent on the availability of acetyl CoA, acetyl CoA and malonyl CoA levels were also measured in the hearts. Low levels of acetyl CoA would favor low ACC activity due to elimination of substrate supply. As shown in Table III.2 acetyl CoA levels were significantly depressed in isoproterenol-treated hearts compared to control hearts. Thus, while the ACC assay shows a non-significant

downward trend in ACC activity, the significantly low levels of acetyl CoA, malonyl CoA and the significant increase in AMPK activity and fatty acid oxidation suggest that malonyl CoA production by ACC *in vivo* was decreased. In addition, the activity of ACC is regulated by phosphorylation from PKA. While direct influence of ACC by PKA, which is highly activated following β-adrenergic stimulation, is unknown in cardiac tissue, the influence of this kinase appears to be limited as a greater decrease in ACC activity would have been expected.

The absence of a shift in energy substrate metabolism from fatty acid oxidation to glucose oxidation can be understood if PDC is immature, and this hypothesis is supported by this study. The activity of PDC in untreated 7-day-old hearts is nearly half the activity measured in adult rabbits *in vivo* (12). Only after isoproterenol administration did PDC activity in the immature hearts reach adult levels (12). In addition, PDC activity in inotrope-treated adult rats is 2-fold greater than PDC activity in inotrope-treated newborn hearts. The low rates of glucose oxidation, basal PDC, as well as stimulated PDC activity from the newborn heart suggest that PDC is immature. Furthermore, the low levels of acetyl CoA lend additional support to PDC function being limited in the neonate. Upon catecholamine treatment TCA cycle flux increases. However, if PDC is unable to produce the quantity of acetyl CoA needed to meet ATP demands a decrease in acetyl CoA will occur. The low acetyl CoA levels, in concert with phosphorylation of ACC would be expected to reduce the activity of ACC. The

resulting decrease in malonyl CoA levels would remove inhibition of CPT 1, leading to increased rates of fatty acid oxidation.

The consequences of high fatty acid oxidation rates become apparent in the ischemic setting. As stated previously, high rates of fatty acid oxidation can suppress glucose oxidation rates as acetyl CoA from fatty acid metabolism may inhibit PDC through activation of pyruvate dehydrogenase kinase. However, glycolytic rates will remain relatively unaffected (13). Research suggests that low rates of glucose oxidation relative to glycolytic rates leads to poor recovery post-ischemia (9,10,14). In light of this, I hypothesize that high levels of fatty acid oxidation produced by catecholamines in the newborn heart may jeopardize post-ischemic functional recovery.

Summary

Glucose oxidation rates and PDC activity in the neonatal heart increase after treatment with a catecholamine. However, basal and stimulated PDC activities and rates of glucose oxidation are very low such that glucose oxidation alone is unable to supply the extra ATP required. As a result, fatty acid oxidation remains the major source of energy in isoproterenol-treated hearts. The stimulation of fatty acid oxidation following inotropic stimulation appears to be due to a dramatic decrease in malonyl CoA levels. In light of these events, I suggest that the pyruvate dehydrogenase complex is immature in the newborn.

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Chapter IV

Isoproterenol Treatment Before or After Severe Ischemia does not Improve Functional Recovery in 7-Day-Old Rabbit Hearts

Introduction

It has been previously shown that following inotropic stimulation an increase in the oxidation of fatty acids provides the majority of energy for newborn rabbit hearts (Chapter III). Isoproterenol did increase the oxidation rates of glucose and activity of PDC. However, relative to fatty acid oxidation, the contribution of acetyl CoA from glucose to the TCA cycle was substantially less.

This is in contrast to the adult heart, where inotropes primarily stimulate glucose oxidation (1-3). In several studies using an adult model of working heart perfusion, treatment with inotropes resulted in a shift of energy production from fatty acid oxidation to glucose oxidation. These studies identified a definite preference for glucose oxidation to meet the inotrope-induced increase in cardiac work. The preference in the newborn heart, as identified in Chapter III, is essentially non-selective such that fatty acid oxidation remains the major contributor of energy.

High fatty acid oxidation rates can be detrimental in the ischemic setting (4-6). High rates of fatty acid oxidation suppress glucose oxidation rates, since acetyl CoA from fatty acid oxidation can inhibit PDC through activation of PDH kinase [reviewed in Stanley *et al.* (7)]. However, despite this decrease in glucose

oxidation, glycolytic rates will remain relatively unaffected (8). During reperfusion, extrusion of H^+, accumulated during ischemia, can induce cell injury via Ca^{2+} overload (9). Therefore, if fatty acid oxidation recovers quickly post-ischemia, the enhanced H^+ production from the uncoupling of glycolysis from glucose oxidation, may exacerbate injury [reviewed in Stanley *et al.* (7)].

Uncoupling of glycolysis from glucose oxidation prior to ischemia has the potential to enhance H^+ production during ischemia. Following isoproterenol administration, high rates of glycolysis (3-fold greater than control) are produced. These high rates of glycolysis may result in an increased breakdown of glycogen during ischemia. This anaerobic glycolysis will lead to more H^+ accumulation and upon reperfusion, higher levels of intracellular Ca^{2+}.

Inotropic agents have been shown to produce negative effects when administered in the peri-operative setting (10). Nonetheless, catecholamine therapy for infants with congenital heart defects is often indicated post-operatively (11). However, in more serious of cases, inotropic treatment may be indicated before surgery. As stated previously, uncoupling of glycolysis from glucose oxidation post-ischemia may exacerbate injury. Therefore, as I have shown that inotropic stimulation significantly increases the oxidation of fatty acids resulting in further uncoupling of glycolysis from glucose oxidation in an aerobic period (Chapter III), I hypothesize that inotropic treatment may also enhance uncoupling during reperfusion. In addition, I also hypothesize that significant uncoupling pre-ischemia may lead to increased lactate and H^+ production during ischemia

resulting in poor functional recovery during reperfusion. For these reasons, I chose to assess the consequences of inotropic treatment both before and after an ischemic insult, on metabolism of energy substrates in, and functional recovery of, newborn hearts.

Materials and Methods

Materials

Refer to Chapter II section 1.

Isolated Heart Perfusions

Methods are as presented in Chapter II.2, with the following amendments. The perfusate contained 1.2 mM palmitate. The perfusion protocol consisted of a 30 min working heart aerobic perfusion, a 30 min global no-flow ischemic period, followed by a 40 min aerobic reperfusion. Isoproterenól, when present, was administered at 5 (Iso 0 min) or 60 min (Iso 60 min) into the working heart perfusion.

Measurements of Glycolysis, Glucose Oxidation, Lactate Oxidation and Fatty Acid Oxidation

As described in Chapter II.3.

Determination of Myocardial Metabolites

As described in Chapter II.5.2 through 5.4.

Statistics

As described in Chapter II.6.

Results

Effects of Isoproterenol on Mechanical Function

The addition of $3 \bullet 10^{-7}$ M isoproterenol in the pre-ischemic period significantly increased most measured parameters of cardiac function (Table IV.1). A significant increase in heart rate, cardiac output, aortic flow and cardiac work (Figure IV.1) was seen in isoproterenol-treated hearts, compared to control hearts, pre-ischemia. No significant changes were seen in peak systolic pressure or coronary flow. During reperfusion, all three groups experienced significantly reduced function in most of the measured parameters with respect to their pre-ischemic values. However, there were no significant differences in function between any of the groups, excluding a reduced peak systolic pressure in Iso 0 min hearts. The percent recovery of cardiac work was highest in the control hearts and lowest in the Iso 0 min hearts, although there were no significant differences in absolute recovery of cardiac work.

Effects of Isoproterenol on Glycolysis, Glucose Oxidation, Lactate Oxidation and Palmitate Oxidation

Cumulative rates of glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation throughout the 100 min perfusion period are shown in Figures IV.2 through IV.5, respectively. As expected, addition of isoproterenol pre-ischemia produced a significant linear increase in rates of glycolysis and glucose oxidation, whereas rates of lactate oxidation and palmitate oxidation did not

change significantly in the pre-ischemic period. Essentially, during reperfusion rates of glycolysis and glucose oxidation were significantly greater in Iso 0 min hearts than in control and Iso 60 min hearts. The exceptions are at 80 min, where Iso 60 min and Iso 0 min hearts were not significantly different from each other, and at 90 min, where all experimental groups were not significantly different from each other. The cumulative rates of glycolysis and glucose oxidation did not differ between control and Iso 60 min hearts throughout the entire perfusion. Cumulative rates of lactate oxidation and palmitate oxidation do not differ statistically between all groups during the entire perfusion.

Steady state rates of glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation are shown in Figures IV.6 through IV.9, respectively. In the pre-ischemic period, Iso 0 min hearts had significantly higher rates of glycolysis (220% of control) and glucose oxidation (387% of control). During reperfusion, rates of glycolysis and glucose oxidation recovered to pre-ischemic values in control hearts. However, isoproterenol did not enhance the metabolism of glucose in either Iso 0 min or Iso 60 min hearts. Rates of lactate oxidation did not change significantly following pre-ischemic isoproterenol administration and were depressed in all groups upon reperfusion. In addition, palmitate oxidation rates did not change significantly after treatment with isoproterenol pre-ischemia or during reperfusion.

Tricarboxylic Acid Cycle Activity

TCA cycle activity (Figures IV.10 and IV.11) was measured as the contribution of acetyl CoA to the TCA cycle from the catabolism of each particular substrate (glucose, palmitate, lactate). In pre-ischemic (Figure IV.10) control hearts fatty acid oxidation contributed 91% of the acetyl CoA for the TCA cycle, while overall carbohydrate oxidation (glucose and lactate) provided only 9%. However, in Iso 0 min hearts, the pre-ischemic percent contribution of acetyl CoA from palmitate oxidation decreased slightly to 85% and carbohydrate oxidation increased to 15%, with respect to control hearts. Despite this pre-ischemic increase in carbohydrate oxidation in Iso 0 min hearts, fatty acid oxidation remained the primary source of TCA cycle acetyl CoA.

During reperfusion (Figure IV.11) the relative contribution of acetyl CoA from carbohydrates and fatty acids were similar between all groups. Carbohydrate oxidation contributed 10% of the acetyl CoA in control hearts and Iso 60 min hearts, and 13 % in Iso 0 min hearts. Fatty acid oxidation contributed 90% of the acetyl CoA in control hearts and Iso 60 min hearts, and 87% in Iso 0 min hearts.

Relative Calculated ATP Production

Figures IV.12 and IV.13 show the contribution of glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation to relative rates of ATP production. In the pre-ischemic period (Figure IV.12), isoproterenol treatment resulted in an increase in the contribution of ATP from glycolysis (10% to 20%) and glucose oxidation (3% to 10%). Lactate oxidation contribution to ATP

production decreased (5% to 3%) and palmitate oxidation contributed relatively less to ATP production (81% to 68%), with respect to control hearts. During reperfusion (Figure IV.13), the relative contribution of ATP was similar in all experimental groups with fatty acid oxidation contributing approximately 70%.

Tissue Metabolites

Lactate levels are presented in Table IV.2. At 30 min, the lactate levels in Iso 0 min hearts were significantly greater than in control hearts. At the end of ischemia, lactate in Iso 0 min hearts was significantly greater than in control hearts, whereas Iso 60 min levels of lactate were not. However, the production of lactate in both control and Iso 0 min groups during ischemia was not significantly different.

Levels of nucleotides (ATP, ADP, ATP/ADP and AMP), creatine (Cr), creatine phosphate (CrP) in hearts frozen after 30, 60 and 100 min of perfusion, are presented in Table IV.3. By in large, there were no significant differences in the levels of these cellular metabolites, with respect to control hearts. However, in both Iso 0 min and Iso 60 min hearts there were significantly lower levels of ATP/ADP and the end of reperfusion.

Hydrogen Ion Accumulation

The levels of H^+ ions pre-, during and post-ischemia, are presented in Figure IV.14. In a subset of hearts (N=5), for which matching glycolysis and glucose oxidation values were available, the initial 30 min of perfusion with isoproterenol resulted in a significant 476% increase (98 to 552 $\mu mol \bullet g\ dry^{-1}$) in

the accumulation of H^+ ions with respect to control hearts. The level of H^+ accumulation during the 30 min ischemia can be estimated from the levels of lactate, as 2 H^+ is produced from the hydrolysis of 2 ATP for each molecule of glucose that passes through glycolysis. The H^+ accumulation in control (N = 5) and Iso 0 min hearts (N = 5) (222 and 165 μmol H^+ • g dry^{-1}, respectively) were not significantly different from each other. During the 40 min reperfusion, H^+ accumulation was significantly greater in Iso 0 min hearts as compared to control hearts. Accumulation of H^+ in Iso 60 hearts was not significantly different from control hearts.

Table IV.1

Isoproterenol Effects on Mechanical Function in Isolated Working Hearts from 7-Day-Old Rabbits

Parameter	Pre-ischemia			Post-ischemia		
	Control (N = 16)	Iso 60 min (N = 11)	Iso 0 min (N = 15)	Control (N = 16)	Iso 60 min (N = 11)	Iso 0 min (N = 15)
Heart Rate (beats•min^{-1})	240 ± 9	255 ± 13	339 ± 6*†	188 ± 20‡	255 ± 49	180 ± 46‡
Peak Systolic Pressure (mmHg)	51 ± 1	48 ± 1	51 ± 1	39 ± 3‡	28 ± 5‡	24 ± 5*‡
Cardiac Output (ml•min^{-1})	30 ± 2	32 ± 2	47 ± 3*†	19 ± 3‡	14 ± 3‡	12 ± 4‡
Coronary Flow (ml•min^{-1})	9 ± 1	9 ± 1	10 ± 1	10 ± 2	7 ± 1	5 ± 1‡
Cardiac Work (ml•min^{-1}•mmHg•10^{-2})	16 ± 1	16 ± 1	24 ± 1*†	8 ± 1‡	6 ± 2‡	5 ± 2‡

Data are mean values of measurements obtained at 30 and 100 minutes after initiation of the working heart perfusion. Values are the mean ± SE.

* Significantly different from control hearts.

† Significantly different from Iso 60 min hearts.

‡ Significantly different from pre-ischemic value.

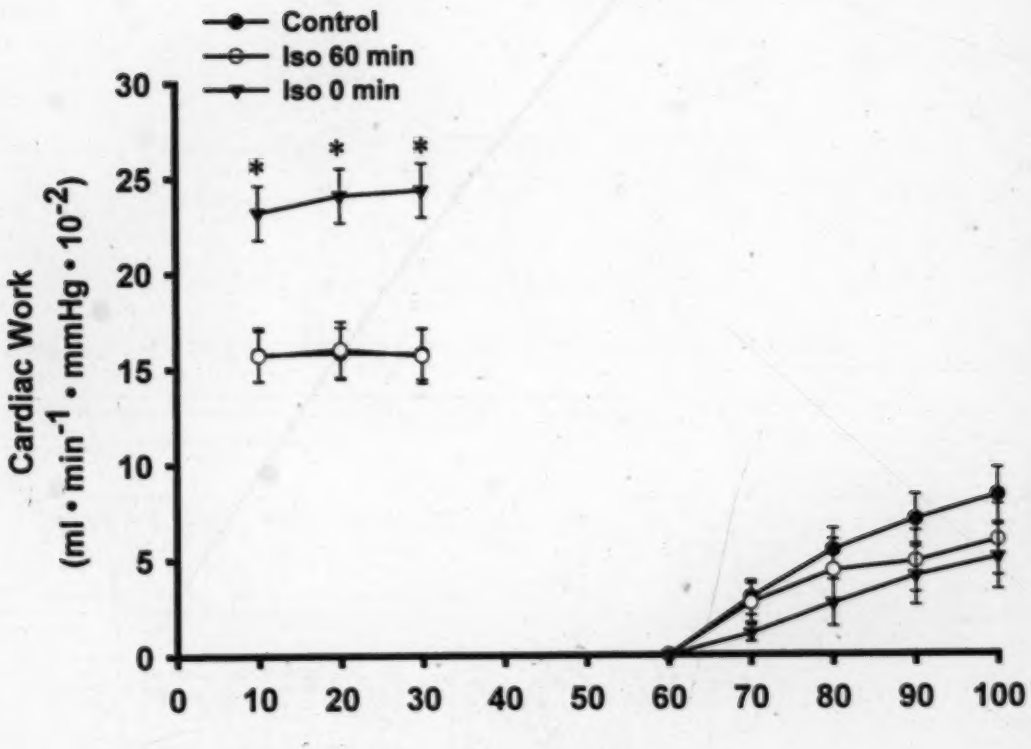

Figure IV.1 Effects of isoproterenol on the time course of cardiac work in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=16; Iso 60 min, N=15; Iso 0 min, N=14.
* Significantly different from Control hearts at the corresponding perfusion time.

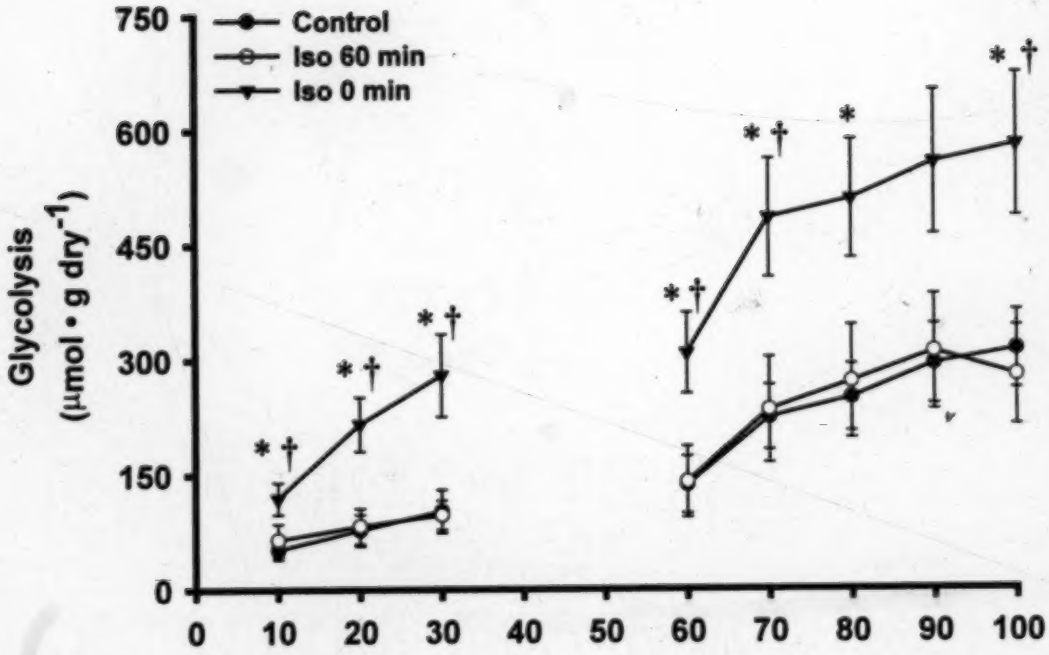

Figure IV.2 Effects of isoproterenol on the time course of glycolysis in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=7; Iso 60 min, N=6; Iso 0 min, N=7.

* Significantly different from Control hearts at the corresponding perfusion time.

† Significantly different from Iso 60 min hearts at the corresponding perfusion time.

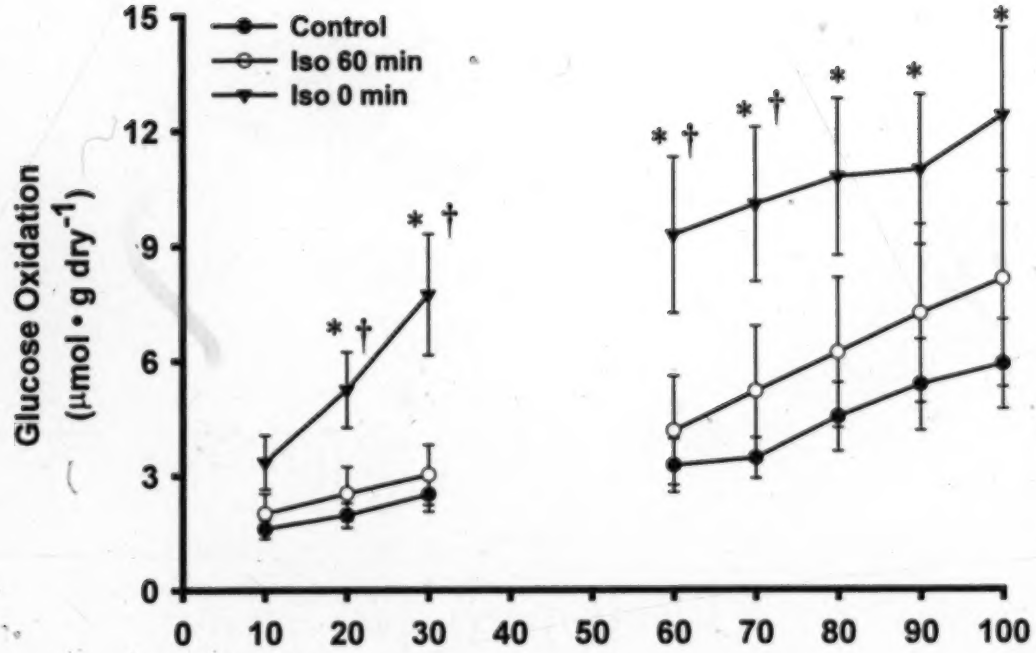

Figure IV.3 Effects of isoproterenol on the time course of glucose oxidation in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=10; Iso 60 min, N=7; Iso 0 min, N=7.
* Significantly different from Control hearts at the corresponding perfusion time.
† Significantly different from Iso 60 min hearts at the corresponding perfusion time.

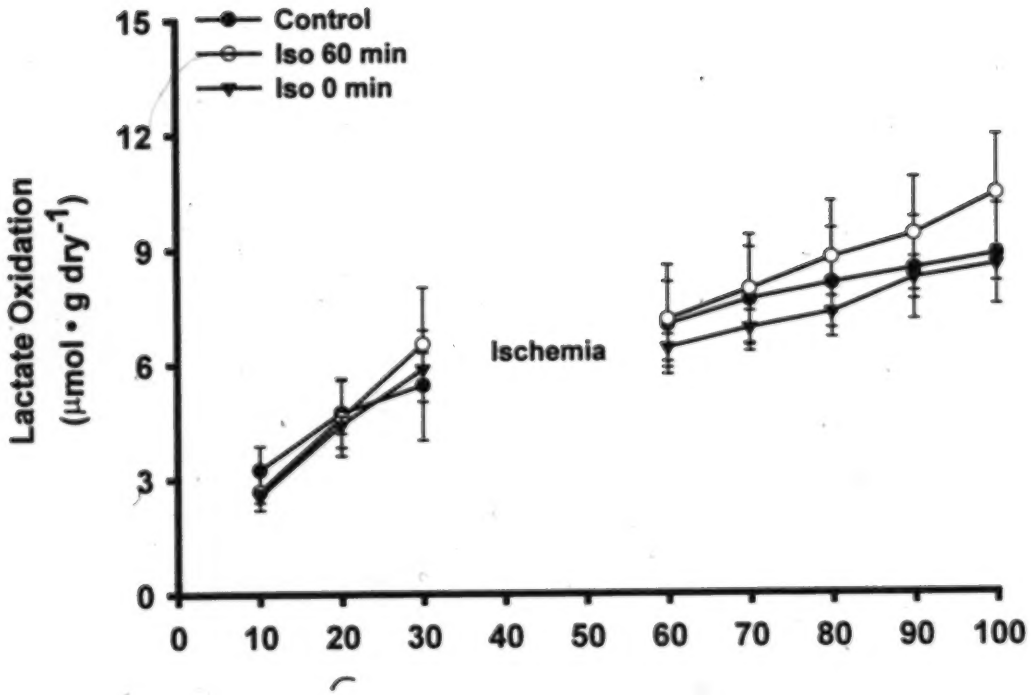

Figure IV.4 Effects of isoproterenol on the time course of lactate oxidation in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=6; Iso 60 min, N=6; Iso 0 min, N=7.

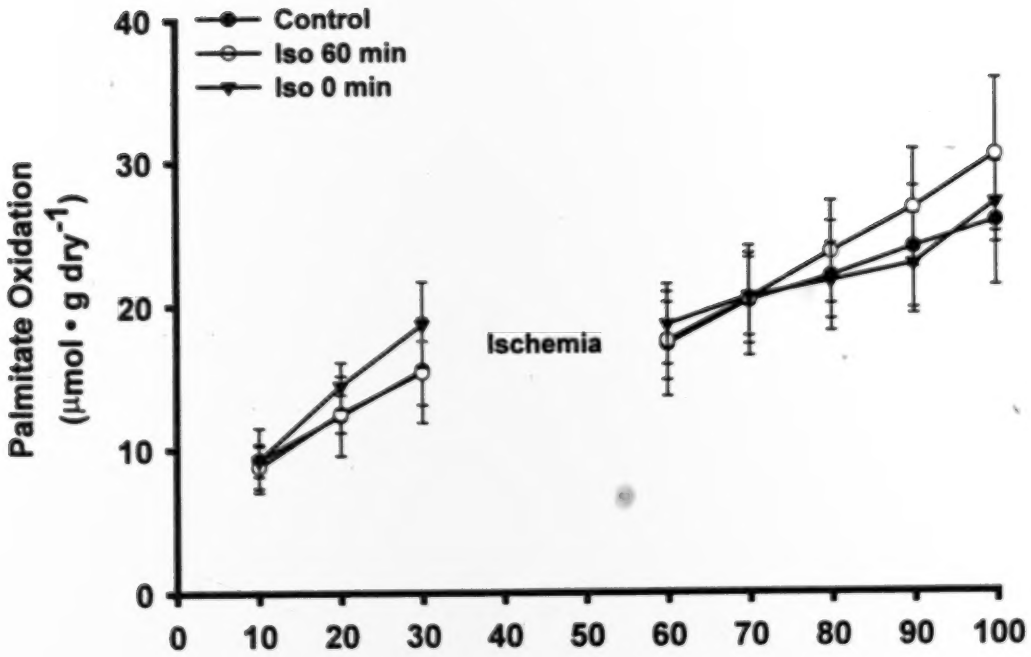

Figure IV.5 Effects of isoproterenol on the time course of palmitate oxidation in isolated working 7-day-old rabbit hearts. Values are the mean ± SE. Control, N=6; Iso 60 min, N=6; Iso 0 min, N=7.

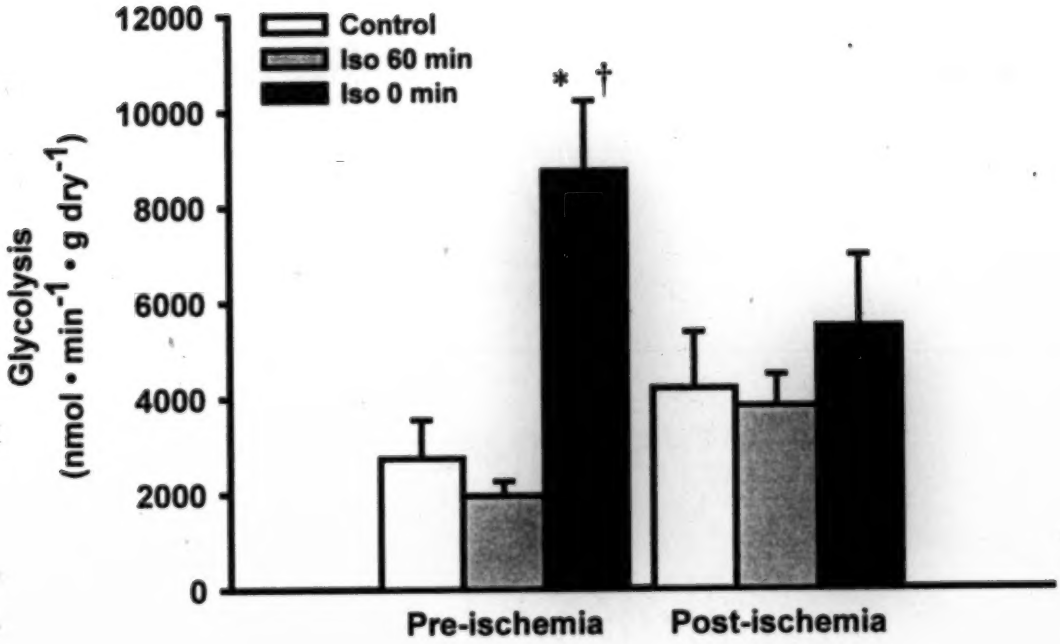

Figure IV.6 Effects of isoproterenol on the steady state rates of glycolysis in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 (Pre-ischemia), 80, 90 and 100 min (Post-ischemia), after initiation of working heart perfusion. Values are the mean ± SE. Control, N = 7; Iso 60 min, N = 6; Iso 0 min, N = 7.

* Significantly different from control hearts.

† Significantly different from Isoproterenol 60 min hearts.

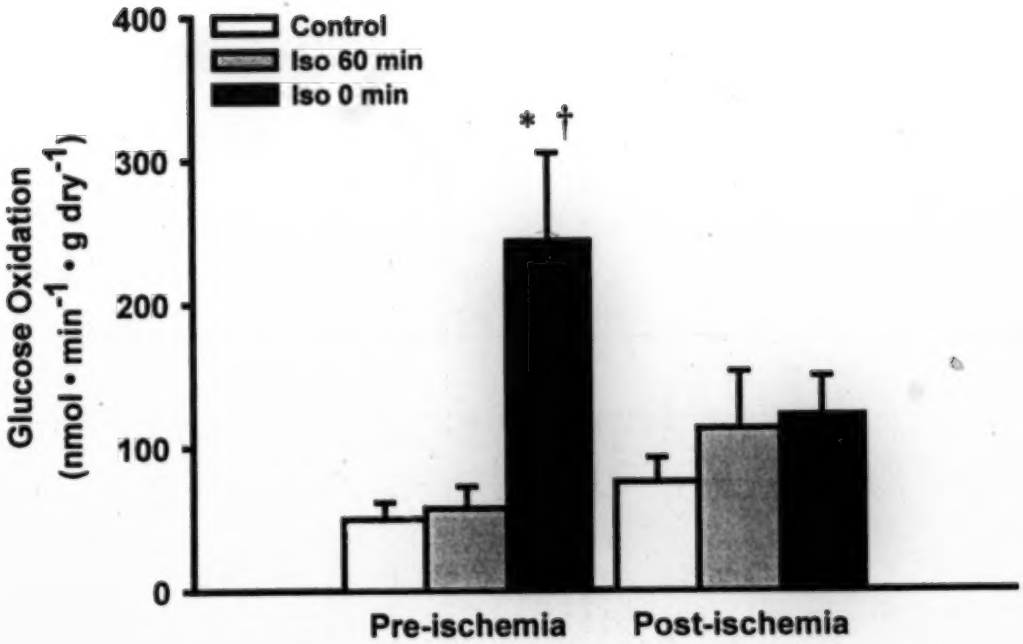

Figure IV.7 Effects of isoproterenol on the steady state rates of glucose oxidation in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 (Pre-ischemia), 70, 80, 90 and 100 min (Post-ischemia), after initiation of working heart perfusion. Values are the mean ± SE. Control, N=10; Iso 60 min, N=7; Iso 0 min, N=7.

* Significantly different from control hearts.

† Significantly different from Isoproterenol 60 min hearts.

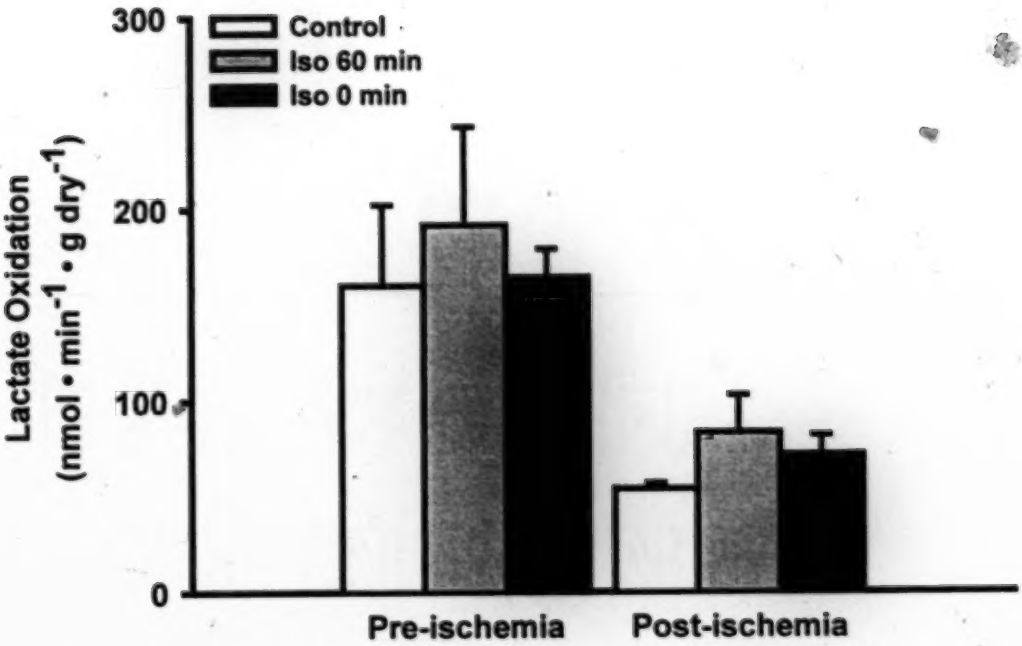

Figure IV.8 Effects of isoproterenol on the steady state rates of lactate oxidation in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 (Pre-ischemia), 70, 80, 90 and 100 min (Post-ischemia), after initiation of working heart perfusion. Values are the mean ± SE. Control, N=6; Iso 60 min, N=6; Iso 0 min, N=7.

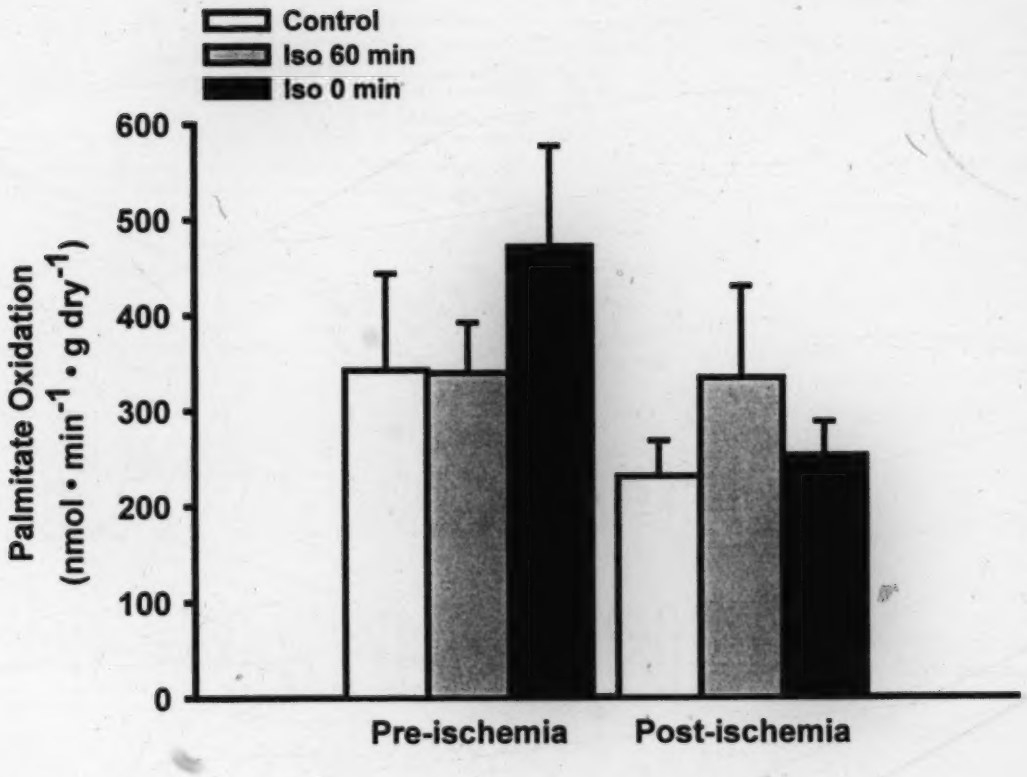

Figure IV.9 Effects of isoproterenol on the steady state rates of palmitate oxidation in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 (Pre-ischemia), 70, 80, 90 and 100 min (Post-ischemia), after initiation of working heart perfusion. Values are the mean ± SE. Control, N=6; Iso 60 min, N=6; Iso 0 min, N=7.

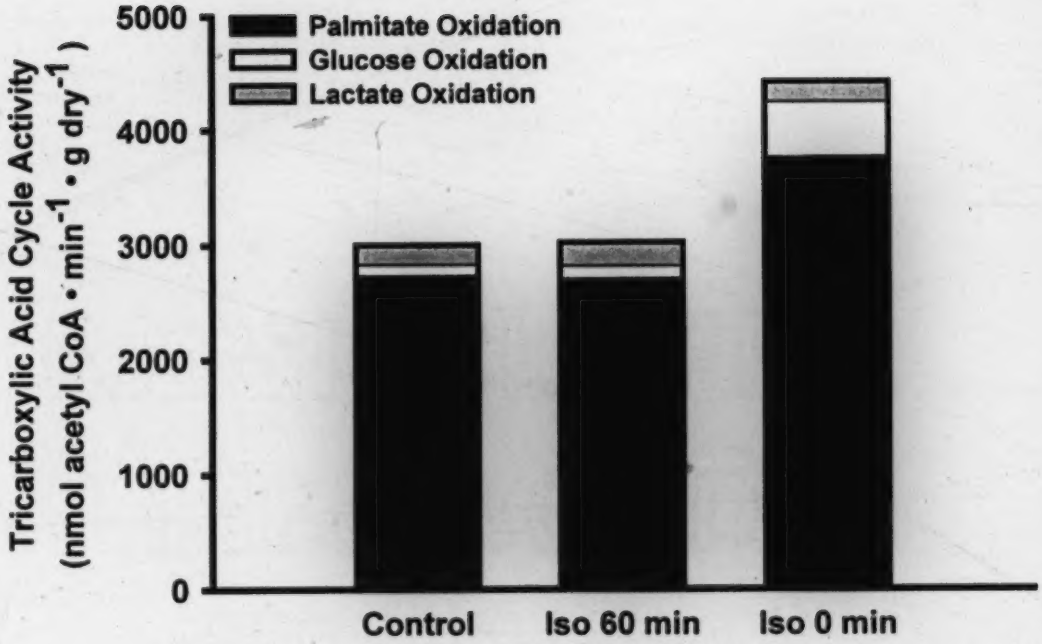

Figure IV.10 Effects of isoproterenol on pre-ischemic absolute rates of acetyl CoA production from glucose oxidation, lactate oxidation and palmitate oxidation in isolated working 7-day-old rabbit hearts.

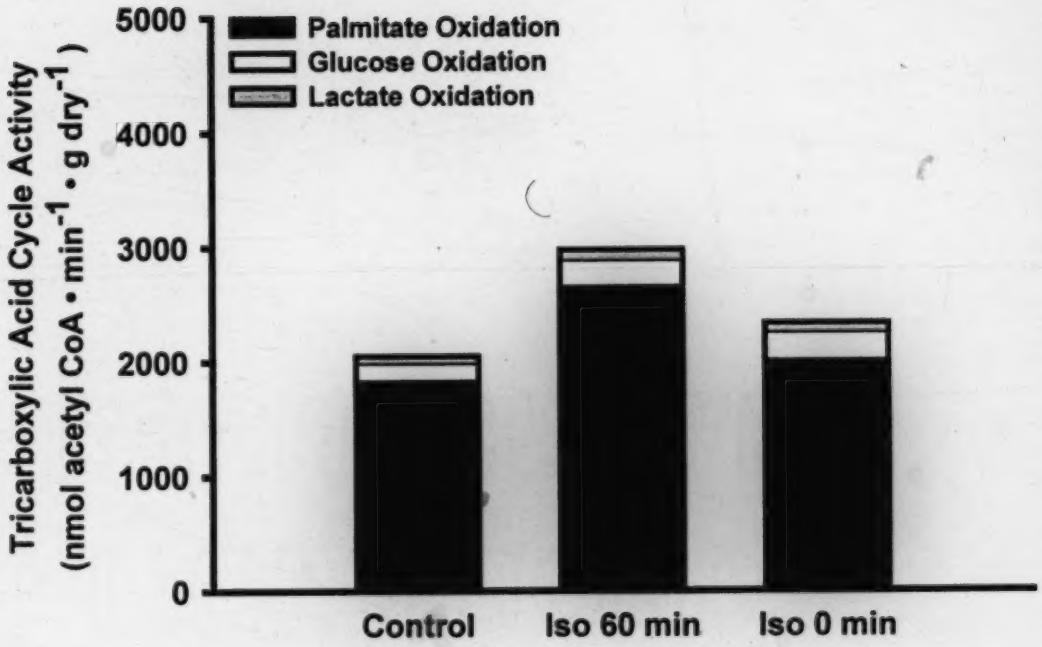

Figure IV.11 Effects of isoproterenol on post-ischemic absolute rates of acetyl CoA production from glucose oxidation, lactate oxidation and palmitate oxidation in isolated working 7-day-old rabbit hearts.

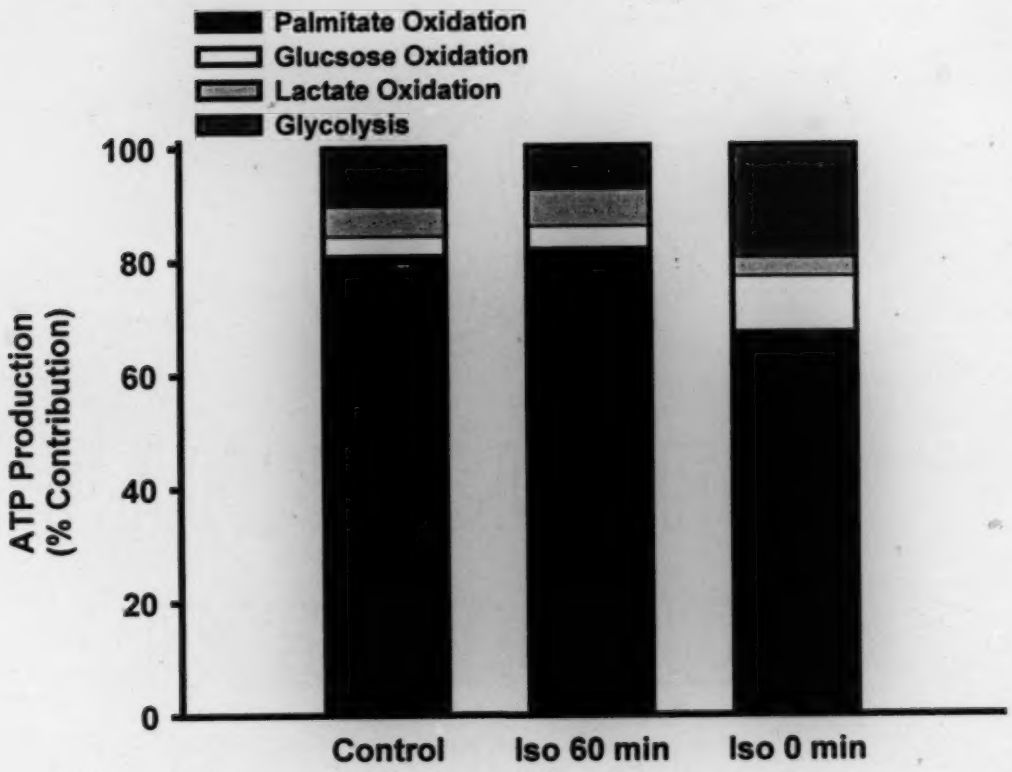

Figure IV.12 Effects of isoproterenol on pre-ischemic relative rates of ATP production from glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation in isolated working 7-day-old rabbit hearts.

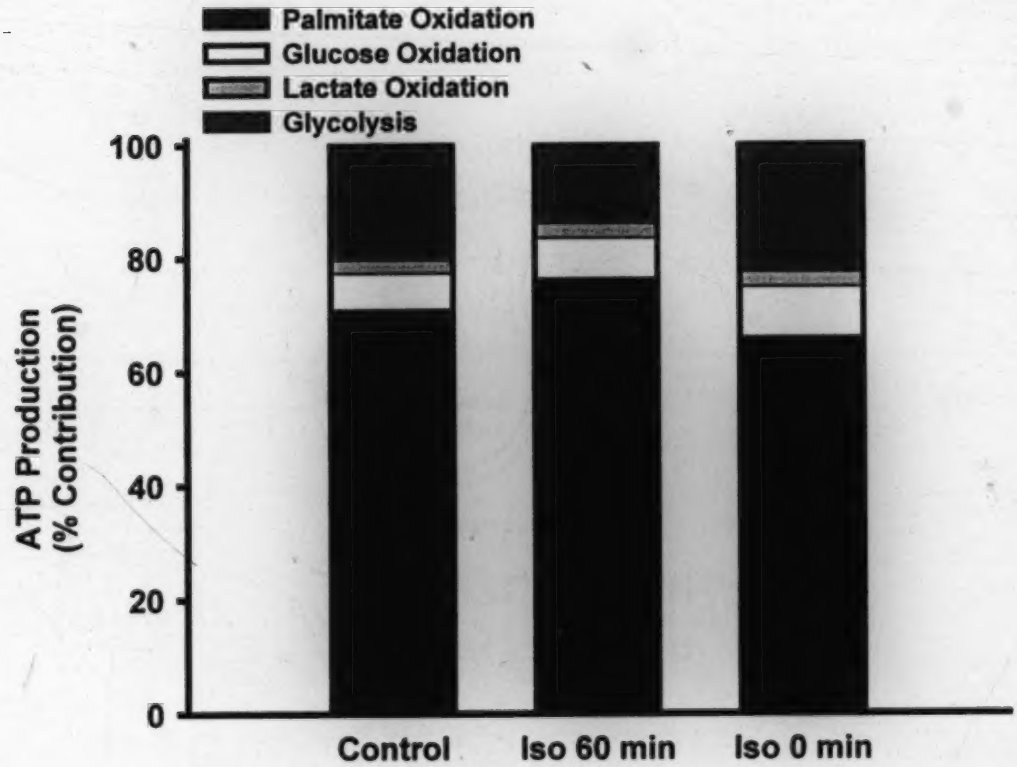

Figure IV.13 Effects of isoproterenol on post-ischemic relative rates of ATP production from glycolysis, glucose oxidation, lactate oxidation and palmitate oxidation in isolated working 7-day-old rabbit hearts.

Table IV.2

Effects of Isoproterenol on Lactate Levels in Isolated Working Hearts from 7-Day-Old Rabbits

	Control			Iso 0 min			Iso 60 min
µmol• $g\ dry^{-1}$	Pre- (N = 5)	Ischemia (N = 6)	Post- (N = 5)	Pre- (N = 6)	Ischemia (N = 5)	Post- (N = 6)	Post- (N = 6)
Lactate	5 ± 1	240 ± 32	18 ± 7	19 ± 3*	183 ± 21	83 ± 3*	46 ± 21

Data are the values of measurements obtained at 30, 60 and 100 min after the initiation of the working heart perfusion. Values are the mean ± SE.

* Significantly different from control hearts.

Table IV.3

Effects of Isoproterenol on Selected Metabolites in Isolated Working Hearts from 7-Day-Old Rabbits

	Control			Iso 0 min			Iso 60 min
µmol• g dry^{-1}	Pre- (N = 6)	Ischemia (N = 6)	Post- (N = 7)	Pre- (N = 5)	Ischemia (N = 6)	Post- (N = 7)	Post- (N = 7)
ATP	25 ± 4	21 ± 4	24 ± 1	24 ± 2	13 ± 2*	15 ± 3	18 ± 3
ADP	15 ± 2	18 ± 2	11 ± 1	18 ± 1	18 ± 1	16 + 2	20 ± 2
ATP/ADP	1.8 + 0.3	1.2 ± 0.2	2.4 ± 0.2	1.3 ± 0.1	0.9 ± 0.1	0.9 ± 0.1*	1.0 ± 0.2*
AMP	2 ± 2	9 ± 3	1 ± 1	4 ± 1	18 ± 3	9 ± 2*	6 ± 2
CrP	48 ± 9	21 ± 1	49 ± 6	30 ± 4	30 ± 4	37 ± 5	35 ± 6
Cr	129 ± 17	214 ± 22	169 ± 25	185 ± 26	267 ± 21	225 ± 21	166 ± 24

Data are the values of measurements obtained at 30, 60 and 100 min after the initiation of the working heart perfusion. Values are the mean ± SE.

* Significantly different from control hearts.

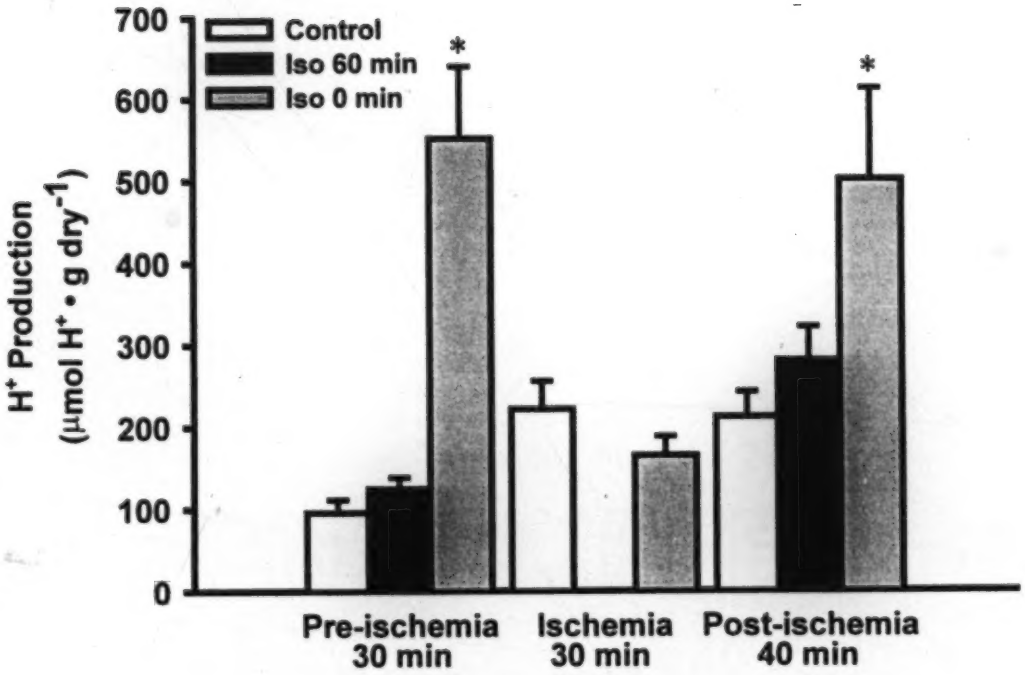

Figure IV.14 Effects of isoproterenol on H^+ production in isolated working 7-day-old rabbit hearts. Data are values of measurements obtained at 20, 30 (Pre-ischemia), 30 and 60 min (Ischemia), 70, 80, 90 and 100 min (Post-ischemia), after initiation of working heart perfusion. Values are the mean ± SE. Control, N=5; Iso 0 min, N=5; Iso 60 min, N=5.

* Significantly different from control hearts.

Discussion

This study was designed to test the hypothesis that isoproterenol treatment pre- and post-ischemia would compromise heart function due to an uncoupling of glycolysis from glucose oxidation, as a result of high rates of fatty acid oxidation [reviewed in Lopaschuk *et al.* (5)]. This study confirms previous research (Chapter III) in which fatty acid oxidation remains the major source of energy in the neonatal myocardium following isoproterenol treatment. In addition, it was observed that fatty acid oxidation remains the major source of energy in all experimental groups after during reperfusion. However, it was also observed there were no significant differences in functional recovery between the three groups. In effect, isoproterenol does not appear to harm or benefit functional recovery.

In spite of my suggestion that a large degree of pre-ischemic uncoupling, after isoproterenol treatment, would result in greater glycogen consumption during ischemia leading to higher levels of lactate, H^+ and subsequent decline in function, the data does not support this original hypothesis. Furthermore, during reperfusion there was no significant difference in rate of glucose oxidation and glycolysis between any of the groups. This suggests that the ability of isoproterenol to enhance metabolism of energy substrates in lost during reperfusion. While there was a significant difference in the accumulation of lactate during reperfusion between control and Iso 0 min hearts, there was no difference in cardiac function. The absence of an observed inotropic stimulation

on cardiac work and metabolism during reperfusion in isoproterenol treated hearts may be a result of Ca^{2+} overload resulting in ischemic damage, or β-AR desensitization. Desensitization of β-AR during ischemia from endogenous catecholamine release has been documented to occur in periods of ischemia lasting 30 min (12,13). However, evidence suggests that agonist-induced desensitization of β-AR does not occur in the newborn (14-17). Nevertheless, it would be prudent and relatively easy to examine for desensitization using such agents as forskolin, dibutyryl-cAMP and by performing a post-ischemic isoproterenol concentration-response curve.

Furthermore, the similarity of ATP, ADP, AMP, creatine and creatine phosphate content between all groups lends further support that isoproterenol administration neither harms, nor benefits newborn hearts in our model.

Summary

Metabolism of free fatty acids is the major source of energy in the newborn myocardium in the presence or absence of inotropes. In addition, inotropic administration before ischemia causes dramatic uncoupling between glycolysis and glucose oxidation. However, during ischemia, H^+ accumulation is not significantly different than in control hearts and uncoupling upon reperfusion appears to be minor. In conclusion, in this model, inotropic treatment before or after severe ischemia is not beneficial for enhancing cardiac work during reperfusion.

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Chapter V

Discussion and Conclusions

Thesis Objectives

The initial objective of this thesis was to determine the effects of isoproterenol treatment on energy substrate metabolism and function in newborn hearts. In addition, I generated a hypothesis if it was observed that fatty acid oxidation remained the major source of energy after isoproterenol treatment. I hypothesized that isoproterenol treatment before or after an ischemic insult would negatively affect functional recovery, due to a fatty acid oxidation induced uncoupling of glycolysis from glucose oxidation. To satisfy this primary objective an initial study was undertaken, in which newborn hearts were perfused in aerobic conditions with low concentrations of fatty acids (0.4 mM palmitate). In turn, to test my hypothesis, a second study was performed in which newborn hearts were perfused through a no-flow ischemia/reperfusion protocol with high concentrations of fatty acids (1.2 mM palmitate).

Conclusions

The objectives of this thesis were completed as detailed in the following conclusions. The primary conclusions of this thesis are that glucose oxidation does increase after treatment with isoproterenol. However, after treatment with isoproterenol, fatty acid oxidation remains the major source of ATP. Furthermore, isoproterenol treatment pre- and post-ischemia does not harm or benefit functional

recovery in 7-day-old rabbit hearts. In effect, fatty acid oxidation did remain the major source of energy after treatment with isoproterenol. However my subsequent hypothesis of a decline in functional recovery from ischemia in isoproterenol treated hearts, as compared to control hearts, has been refuted.

Fatty Acid Oxidation Remains the Major Source of Energy Before and After Treatment with Isoproterenol

In this study it is determined that fatty acid oxidation remains the major source of energy for the heart before and after treatment with isoproterenol. While there is an increase in the metabolism of all substrates examined, PDC activity and glucose oxidation were very low before and after catecholamine addition. Unlike the adult rat heart (1-3) treatment with inotropes resulted in increased fatty acid oxidation rates in the neonatal rabbit heart. During the first days of life, the newborn myocardium is clearly in a state of immaturity, undergoing dramatic changes in energy substrate preference. It has been previously shown that 1-day-old rabbits obtain 57% of their ATP from glucose and 43% from fatty acid oxidation, while 7-day-old hearts receive 10% of their ATP from glucose and 90% from fatty acid oxidation (4). This thesis support theses findings showing that fatty acid oxidation provides the majority of energy required by 7-day-old rabbits hearts. As newborns undergo dramatic shifts in energy substrate utilization over the course of several days, it is logical to suggest that key regulatory enzymes may behave differently relative to the mature heart. This suggestion is based on previously observed of developmental changes which occur in several regulatory

enzymes. For example, the expression and activity of AMPK increase over the first week of life (5) and the expression of CPT 1 changes with age to an isoform that has a higher affinity for L-carnitine and is less sensitive to malonyl CoA [reviewed in Makinde *et al.* (6)]. In addition, these enzymes are believed to be responsible, in part, for the large increase in fatty acid oxidation seen in newborn hearts after catecholamine administration.

The basal levels of, and the dramatic increase in, fatty acid oxidation after catecholamine administration were greater than expected considering the relatively low levels of fatty acids in the perfusate. Other research performed in adult hearts reveals strikingly different results than those observed in this study. In the presence of low fatty acid concentrations (0.4 mM palmitate/oleate), in which glucose oxidation is favored, inotrope administration produces dramatic increases in carbohydrate metabolism (1-3). For example, in a study by Collins-Nakai *et al.* epinephrine treatment resulted in a 310% increase in rates glucose oxidation and a 156% increase in the contribution of glucose metabolism to ATP production (1). Furthermore, if hearts are perfused with high fatty acid concentrations (1.2 mM palmitate), which can decrease basal levels of glucose oxidation, inotropic stimulation continued to produce a nearly 3-fold increase in glucose metabolism (7). The result of an increase in glucose metabolism is a shift in the percent contribution of energy from fatty acid oxidation to glycolysis and glucose oxidation. In this study, inotropic administration resulted in virtually no shift in

the source of energy production, with fatty acid oxidation remaining the predominant contributor of acetyl CoA for the TCA cycle.

The rates of fatty acid oxidation in the heart are primarily regulated by phosphorylation control of, and substrate supply to, specific enzymes. CPT 1 is the enzyme responsible for the uptake of fatty acids into the mitochondria. As previously mentioned, CPT 1 is very sensitive to inhibition by malonyl CoA. The activity of ACC, which produces malonyl CoA from acetyl CoA, is primarily dependent on phosphorylation control from AMPK, the availability of acetyl CoA and potential phosphorylation from PKA. In this study, there was a dramatic drop in malonyl CoA levels resulting in increased rates of fatty acid oxidation. The increase in fatty acid oxidation rates produced by isoproterenol was accompanied by an increased activity of AMPK. This increase in AMPK activity will result in phosphorylation and inactivation of ACC (8). Nevertheless, there are two hypotheses that can explain the underlying mechanisms which resulted in fatty acid oxidation remaining the major source of energy after treatment with isoproterenol.

One hypothesis suggests high fatty acid oxidation rates suppress rates of glucose oxidation through activation of pyruvate dehydrogenase kinase. Pyruvate dehydrogenase kinase, which phosphorylates and inactivates PDC, can be activated by acetyl CoA derived from fatty acid oxidation. An opposing hypothesis suggests glucose oxidation is limited in ability to produce the acetyl CoA required to meet the ATP demands of the cell. The resulting decline in

cellular acetyl CoA and ATP levels results in inhibition of ACC, declining malonyl CoA levels, removal of CPT 1 inhibition and subsequent fatty acid oxidation stimulation. The latter hypothesis assumes that PDC is "immature." In this context, the term "immature" defines a developmental difference between newborn and adult PDC function. Whether or not the difference in PDC function arises from discrepancies in PDC expression, activity, or both, it is unknown. Nonetheless, the hypothesis of an immature PDC appears to be the more reasonable of the two proposed mechanisms.

Isoproterenol Treatment Pre- And Post-Ischemia Does Not Harm Or Benefit Functional Recovery In 7-Day-Old Rabbit Hearts

This study confirms previous research in which inotrope administration produces significant increases cardiac work (Chapter III). However, the stimulatory effects of isoproterenol (pre- and post-ischemia) on cardiac work were ameliorated during reperfusion. Furthermore, there were no significant differences in functional recovery between the three groups suggesting that while isoproterenol does not appear to harm functional recovery, it does not appear to be advantageous either.

In addition, this study reproduces previous findings in which fatty acid oxidation remains the major source of energy in the neonatal myocardium after isoproterenol treatment (Chapter III). Furthermore, this study shows that not only does fatty acid oxidation remain the major source of energy after treatment with an inotrope in the aerobic setting, fatty acid oxidation remains the major source of

energy after an ischemic insult in both inotrope-treated and control newborn hearts. It is important to note that all hearts were perfused with high concentrations of fatty acids (1.2 mM palmitate) to reflect serum levels of fatty acids in the surgical setting (9). Serum levels of free fatty acids rise in the peri-operative period in response to heparin activation of lipoprotein lipase [reviewed by Tan (10)], as well as endogenous and exogenous catecholamine activation of hormone sensitive lipases (11). The high levels of circulating free fatty acids dramatically effect the energy substrate utilization of other substrates in the myocardium. For example, perfusion with 1.2 mM palmitate results in a greater decrease in rates of glucose oxidation than and glycolysis, relative to hearts perfused in the presence of low fat (0.4 mM palmitate) (7). My data confirms these trends. Rates of glucose oxidation are 49% lower in this study than in the previous study using low concentrations of fatty acids (Chapter III). Rates of glycolysis, on the other hand, are only 4% lower.

The uncoupling of glycolysis from glucose oxidation in hearts treated with catecholamines pre-ischemia is very significant. The significantly higher rate of glycolysis after inotropic treatment, one might suggest, would result in greater glycogen consumption, both before and during ischemia, and higher levels of H^+ accumulation during ischemia, than in control hearts. As stated previously, I hypothesized that this enhanced calculated level of H^+ production could result in intracellular Ca^{2+} buildup upon reperfusion and cause poor functional recovery. While these data confirm the presence of enhanced lactate accumulation in

isoproterenol treated hearts before ischemia, during ischemia lactate production does not differ between control an Iso 0 min hearts. While early into ischemia the momentum of glycolysis may consume greater levels of glycogen, these data show that lactate production during ischemia in control and Iso 0 min hearts is not significantly different at the end of reperfusion. To explain this finding, I suggest that enhanced glycogenolysis before ischemia, which is observed after treatment with inotropes (12-14), results in lower glycogen levels at the onset of ischemia, relative to control. Therefore, there will be less accumulation of glycolytic products (lactate, H^+) at the end of ischemia than in control hearts.

During reperfusion, there was no significant difference in the steady state rates of glycolysis and glucose oxidation between control, Iso 0 min, and Iso 60 min hearts. Thus, the stimulation of glycolysis and glucose oxidation seen pre-ischemia, following isoproterenol administration, is lost during reperfusion. Furthermore, the similar levels of cellular metabolites (ATP, ADP, AMP, creatine and creatine phosphate) in all groups suggests that, in this model, isoproterenol administration is neither harmful nor of any benefit to newborn hearts.

The inability of isoproterenol to enhance cardiac function during reperfusion does not appear to be dependent on the presence of isoproterenol pre-ischemia. Furthermore, the absence of an increase in the steady state rates of glycolysis post-ischemia suggests that this too, is independent of isoproterenol administration. Rather, the ischemic injury itself and/or possible adrenergic desensitization appears to be responsible for amelioration of inotropic stimulation

of cardiac function. As a result of ischemia and subsequent cell damage, Ca^{2+} overload and elevated levels of cAMP may be such that isoproterenol can not enhance contractility or glycolysis.

Furthermore, endogenous catecholamine release during ischemia has been shown to result in β-AR desensitization upon reperfusion (15,16). The desensitization is not believed to be at the receptor level, but elsewhere such as adenylate cyclase. This is because levels of β-AR in the cell membrane actually increase during ischemia as result of reduced ATP-dependent receptor internalization. While it has also been reported that adenylate cyclase activity is enhanced in early ischemia (≈ 15 min), during prolonged ischemia (≥ 30 min) adenylate cyclase desensitization develops (15). However, adrenergic innervation of the rabbit myocardium does not occur until the 3rd week of life (17). In adults, endogenous catecholamines are released during ischemia from pre-synaptic terminals. Therefore, as innervation would not have occurred by 7-days, it is unlikely that β-AR desensitization would occur in control hearts. Furthermore, as mentioned previously, there is evidence suggesting that newborns experience little or no agonist-induced desensitization (18-21). For example, in 6-day-old rats treatment with isoproterenol actually results in sensitization of β-AR responses (22). Therefore, Iso 0 min hearts, which were exposed to isoproterenol during reperfusion, are unlikely to have experienced β-AR desensitization. Nevertheless, a test for homologous and heterologous desensitization could be performed using

agents including forskolin and dibutyryl-cAMP and by performing a post-ischemia isoproterenol concentration-response curve.

Moreover, the hypothesis of an immature PDC is consistent in both studies. Initially, it may appear that an immature PDC is not compatible with the ischemia/reperfusion study because isoproterenol treatment pre-ischemia results in an increase in glucose oxidation without any increase in fatty acid oxidation. However, the presence of high fatty acid concentrations must be taken into account. The presence of 1.2 mM palmitate in the perfusate resulted in elevated basal levels of fatty acid oxidation (similar to levels seen in isoproterenol treated hearts perfused with low fat) and decreased basal levels of glucose oxidation. Isoproterenol treatment produced an elevation in rates of pre-ischemic glucose oxidation to levels similar to levels of glucose oxidation in isoproterenol treated hearts perfused with low concentrations of fatty acids. In effect, hearts perfused in the presence of high fatty acids begin at a different baseline levels of metabolism (elevated rates of fatty acid oxidation and reduced glucose oxidation rates). However, after isoproterenol treatment the metabolic profile of hearts perfused in both low and high concentrations of fatty acids becomes very similar.

Summary

Fatty acid oxidation remains the major source of ATP before and after treatment with isoproterenol, and isoproterenol treatment pre- and post-ischemia does not harm or benefit functional recovery in 7-day-old rabbit hearts. Fatty acid oxidation remaining the major source of energy contribution before and after

isoproterenol treatment can be attributed to PDC immaturity. The absence of enhanced injury in isoproterenol treated hearts pre- and post-ischemia may be attributed to similar glycolysis rates during ischemia and similar rates of glycolysis from glucose oxidation during reperfusion, with respect to control hearts.

Limitations

One limitation of this thesis is the absence of O_2 consumption data. The measurement of O_2 consumption in the 7-day-old newborn rabbit hearts would permit calculation of MVO_2 and efficiency. As inotropes have been considered to be "O_2 wasting" (23,24) this data would help to clarify whether or not O_2 use matches cardiac work after inotrope administration in newborn hearts. Previous work in adult hearts suggests that if inotropes are "O_2 wasting" this must occur after ATP production (1). The authors point out that O_2 consumption matches the increase in cardiac work and that this is a direct result of glucose oxidation selectively increasing to meet the new energy demand. They further suggest that if fatty acid oxidation had increased instead the O_2 consumption per cardiac work would have increase resulting in decreased efficiency. Nonetheless, during the course of my research numerous attempts had been made to obtain reproducible and accurate O_2 measurements. Unfortunately, these efforts were in vain as reliable O_2 consumption data could not be produced.

Another limitation is the absence of protein expression data for PDC in 7-day-old and mature rabbits. This data would allow for a more conclusive analysis of

PDC behavior in the newborn heart. My hypothesis of an immature PDC resulting in the non-selective increase in metabolism of all energy substrates examined, as opposed to a selective increase in glucose oxidation, is limited. While the activity of PDC is lower than in adult rabbits (25), whether this is a result of regulation or expression is not known.

The absence of data indicating tissue glycogen levels is another limitation of this study. Tissue glycogen levels could provide evidence supporting or refuting the lactate accumulation data during ischemia. Furthermore, they would permit analysis of the inotropic effects on tissue glycogen levels during periods of aerobic perfusion. In summary, these data would help to clarify the effects of inotropes on energy substrate metabolism in the newborn heart and aid in the interpretation of the results. During the course of my research the glycogen assay had been performed, however, inconsistency of the results precluded their inclusion in this study.

Future Directions

The experimental model used in this thesis subjects the myocardium to a severe 30 min global no-flow ischemic insult. The limitation of producing a severe injury is that any subtle deleterious effects of isoproterenol will be masked by the overriding ischemic insult. Clinically, the use of cardioplegia and whole body cooling lessens the deleterious effects of ischemia. In such instances, any negative effects of inotropic support in the peri-operative period may precipitate, unmasked by the less severe ischemic injury. In my experiments, the control

recovery was less than 50%. To determine if inotropes may contribute to ischemic injury under a less severe ischemic injury, further experimentation would be necessary. The duration of ischemia could be reduced to a point where control hearts can manage near full recovery. Clearly, it is possible that under a less severe ischemic insult isoproterenol may produce observable deleterious effects.

In addition, isoproterenol is a selective β-adrenergic agonist not typically used in the clinical setting due to frequent tachycardia, arrhythmias, and systemic vasodilation (26). However, as mentioned previously, the β-AR selectivity of isoproterenol was preferred in this thesis to examine the role of ACC and AMPK. Both ACC and AMPK may be phosphorylated by PKA, which is activated following β-AR stimulation. The absence of α-AR stimulation removes confounding variables permitting clearer examination of underlying cellular mechanisms. Nevertheless, as both β and α agonists are typically employed clinically, the use of α- (phenylephrine) and dual agonists (epinephrine) would clarify the pharmacological effects of catecholamines of heart metabolism and function in the ischemia/reperfusion setting.

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